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Obituary Notice

JOHN MASSON GULLAND, 1898–1947

British science sustained a grievous loss by the tragic death of Prof. J. M. Gulland in a railway accident at Goswick, near Berwick-on-Tweed, on 26 October 1947. He was a biochemist whose work was founded on a solid basis of achievement in the field of pure organic chemistry, and he will be remembered above all for his work on nucleic acids. His contributions to the chemistry and biochemistry of these important cell constituents have greatly enriched our knowledge of them and will rank very highly among the pioneer investigations in this field. Gulland was a man of vigour and vivacious charm, of whom Dr Jesse P. Greenstein has written (*Nature, Lond.*, 1948, **161**, 87): 'Gulland was one of a group of distinguished foreign guests at the Cold Spring Harbor Symposium held in June 1947. He was easily the dominant figure at that conference, and the charm and ease of his manner, his gentle critical spirit, together with the scholarliness and incisiveness of his thinking, evoked general admiration and affection.' This spontaneous tribute from across the Atlantic describes Gulland in terms which recall very vividly the man whom his friends admired and respected.

John Masson Gulland was born in Edinburgh in 1898 and was the only son of the late Prof. G. Lovell Gulland, Professor of Medicine in the University of Edinburgh. He was devoted to his native city and had an intense love of Scotland. He had a wide and detailed knowledge of the Scottish Highlands, their local customs, history and geology, and he made some delightful water-colour sketches during his many holidays there. He was also a keen fisherman in Scottish rivers. Gulland was educated at Edinburgh Academy and the University of Edinburgh, where he graduated in 1921, after serving as a second lieutenant in the First World War. Having been awarded a Carnegie Scholarship, he went to work at St Andrews under Prof. (later Sir) Robert Robinson, whom he rejoined in Manchester; again later in Oxford Gulland was in the Dyson Perrins laboratory when Robinson was appointed to the chair of chemistry. Gulland became a University demonstrator in chemistry at Oxford in 1924, and was appointed lecturer in chemistry at Balliol College in 1926. These appointments he relinquished in 1931, when he became senior assistant in biochemistry at the Lister Institute, London, and a reader in biochemistry in the University of London. In 1936, Gulland was appointed to the Sir Jesse Boot Chair of Chemistry at University College, Nottingham. He gave up this post a few weeks before his death to take up a new appointment as director of research to the Institute of Brewing. He was elected a Fellow of the Royal Society in 1945, and the following year he was appointed a representative of the Royal Society on the British National Committee for Chemistry. He was M.A. of Oxford, Ph.D. of St Andrews, D.Sc. of Edinburgh, and a Fellow of the Royal Society of Edinburgh and the Royal Institute of Chemistry. In 1924 he married Ruth Madeline Ida Russell, also an Edinburgh graduate and daughter of the late Sir James A. Russell, and is survived by his wife and two daughters.

Although he has a large amount of first-rate scientific achievement to his credit, Gulland was not quite so prolific a contributor to the original literature as some of his contemporaries. This is partly due to the formidable nature of the problems which he tackled; he never engaged in trivialities. It is probably due also to the fact that his earlier ardour for work at the bench tended to be subordinated later to his interest in organization and administration, for which he had a real flair. He served on the councils of the Chemical Society and the Royal Institute of Chemistry, and was an honorary secretary of the Chemical Society during 1933–6. From 1932 until 1934, he was secretary of Section B of the British Association, and during 1935–7, he was recorder to the Section. In the early years of the Second World War he was senior gas adviser to the North Midland Region of the Ministry of Home Security, and during 1943–4 he was assistant director, Chemical Research and Development, Ministry of Supply. In this capacity he was concerned with the organization and instigation of research and development by firms, research associations, government establishments and universities on a variety of aspects of such topics as general chemicals and explosives, flax, camouflage, adaptation of army equipment of all kinds for use in the tropics, plastics, textiles, paper and rubber. It was in this period also that he was attracted by the potentialities of alginic acid as a chemical raw material, and he took an important part in the establishment of the Scottish Seaweed Research Association. He became a member of its Board of Management, and Chairman of its General Purposes and Chemical Advisory Committees. He was instrumental in forming the Lace Research Council, of which he was a member, and was an independent member of the Board of Trade Working Party on the Lace Industry, appointed in 1946.

A full appreciation of Gulland's scientific work cannot be given here and a general indication of some of its main trends must suffice. During his St Andrews and Manchester days, he worked on the morphine group of alkaloids, and the constitution which is now generally accepted for morphine was first adumbrated in two classical papers, published in 1923 and 1925, by Gulland and Robinson. With R. D. Haworth, Gulland published a series of papers on the aporphine group of *iso*quinoline alkaloids (1928–9). New methods were devised by which were synthesized, among other compounds, bulbocapnine methyl ether and corytuberine dimethyl ether. Gulland also made other chemical studies of compounds of biological interest, including pellitorine, the pungent principle of *Anacyclus pyrethrum*, and the active constituents of *Taxus baccata* (1930–1). His work then followed a more definite biochemical course. With R. A. Peters, in 1929, he investigated the antineuritic properties claimed for certain quinoline and iminazole derivatives and showed that the compounds in question were inactive. Gulland and Peters also showed that pigeons' blood is much richer in reducing substances than is mammalian blood and found that this is due mainly to sulphydryl compounds; ergothioneine hydrochloride was isolated. In the course of an examination of the spermicidal activity of various quinones and aromatic aldehydes and their derivatives, Gulland found that certain aromatic aldehydes were highly effective spermicidal agents, and he showed how their efficiency could be correlated with some of their chemical reactivities and physico-chemical properties. Between 1932 and 1940, Gulland published a series of papers, in conjunction with T. F. Macrae and other collaborators, on the oxytocic principle of the posterior lobe of the pituitary. He described methods of purification and assay of the hormone, and elucidated something of the chemical nature and active groups by studying the action of nitrous and nitric acids, of oxidizing and reducing agents, and of various preparations of proteolytic enzymes. He was able to show that the oxytocic principle represents an oxidation-reduction system, but it did not appear to contain peptide linkages. Some synthetic esters of choline had analogous properties, but the activity was very slight.

In 1933 Gulland and Macrae published the first of a series of papers on purine nucleosides. These compounds, with the related nucleic acids, formed the subject of Gulland's main research interests thereafter. His contributions in this important field were noteworthy, not only for their intrinsic value, but also because of the interest which they stimulated in other laboratories. He has himself summarized some of this work in three reviews (Gulland, *J. chem. Soc.* 1938, p. 1722; 1944, p. 208; Gulland, Barker & Jordan, *Ann. Rev. Biochem.* 1945, **14**, 175). By using ultraviolet absorption spectroscopy, Gulland and his collaborators showed the points of attachment of the sugar residues to the bases in the nucleosides, and his conclusions were substantiated by similar measurements on synthetic products by Kenner, Lythgoe and Todd. Again, in the course of a series of investigations on yeast nucleic acid, Gulland (with G. R. Barker) confirmed the identity of the sugar component as D-ribose. Among his last publications was one in this *Journal* (1948, **42**, 308) on the nature of the reaction of nucleic acids with mustard gas, and a series of three papers in the *Journal of the Chemical Society* (1947, pp. 1129, 1131, 1141) on the deoxypentose nucleic acid of calf thymus. It is a great loss to British science that his investigations in a field of such supreme biological importance should have been brought to a sad and premature close. It is certain, however, that other workers will come forward to build upon the foundations which he has laid, and there can be no doubt that this is the memorial to his work which he himself would have desired.

(The writer is glad to acknowledge his indebtedness to Mrs J. M. Gulland for some of the personal details, and to the Editors of *Nature* for permission to use as a basis for this tribute a somewhat shorter notice which he contributed to *Nature*.)

J. W. COOK

Studies on the Activation and Purification of Blood Fibrinolysin

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(*Received* 26 *November* 1947)

The presence, in normal plasma or serum, of a fibrinolytic agent that can be activated by chloroform, was first reported by Delezenne & Pozerski (1903 *a*, *b*). This effect of chloroform was further investigated by Nolf (1905, 1908) who also observed activation of this fibrinolytic capacity of the blood in dogs given peptone intravenously, provided the liver was excluded from circulation either by clamping the thoracic aorta and inferior vena cava ('thoracic animal'), or simply by interrupting the circulation through the liver by manual compression of the vessels going into the organ. More recently, Tagnon (1942) showed that the activity of this fibrinolytic enzyme was connected with the euglobulin fraction of the blood, since it might be fully recovered by isoelectric precipitation (pH 5·5) after dilution of the plasma with 10 vol. of distilled water. A comparison of this fibrinolytic factor with the so-called 'lytic factor' described by Milstone (1941) as the effective fibrinolytic agent of material isolated from certain strains of β-haemolytic streptococci (streptokinase), was undertaken by Kaplan (1944), Christensen (1944–5), and Christensen & MacLeod (1944–5). Confirmatory evidence of identity appears in several papers (e.g. Macfarlane & Pilling, 1946; Loomis & Smith, 1946).

Further reports have stressed the importance of the activation of this fibrinolytic enzyme in several physiological and pathological conditions. Using the 'fibrinolytic protamine test', it has been shown (Rocha e Silva, Andrade & Teixeira, 1946) that activation of fibrinolysin occurs in anaphylactic and peptone shock, and that a definite relationship seems to exist between the appearance of the fibrinolytic effect and other characteristics of anaphylactic and peptone shock. In traumatic and burn shock also there is a spontaneous activation of the fibrinolytic enzyme of the blood, the intensity of which bears some relationship to the gravity of the shock condition (Tagnon, Levenson, Davidson & Taylor, 1946). Using normal and sensitized guinea pigs, Ungar (1947) demonstrated activation of a fibrinolytic enzyme when pieces of certain organs (liver and lung) were placed *in vitro* in contact respectively with peptone or with the homologous antigen. Macfarlane & Biggs (1946), using the technique of Macfarlane & Pilling (1946), reported activation of plasma fibrinolysin in a number of subjects from a hospital population including cases awaiting operation, undergoing pretreatment and after actual operation, also some accident and miscellaneous pathological cases. A recent report by Ferguson, Travis & Gerheim (1947) ascribes a weak trypsin-like thromboplastic activity to the fibrinolytic enzyme present in normal blood, suggesting participation of this proteolytic enzyme (plasma tryptase) in blood coagulation. The possibility that activation of this enzyme may play a role in the liberation of histamine was recently suggested by Scroggie, Jaques & Rocha e Silva (1947) on the basis of the fact that a trypsin inhibitor from soya beans partially inhibits the release of histamine from cells to plasma when peptone is added to rabbit blood.

The desirability of securing highly active preparations of this fibrinolytic enzyme, in order to study more thoroughly its biological properties, was the main reason for undertaking this study. When the experiments were nearing completion, a paper appeared by Loomis, George & Ryder (1947) in which they described a method for purification of blood fibrinolysin by combining fractionation by ammonium sulphate with several isoelectric precipitations in high dilutions with distilled water. Under present circumstances, we cannot undertake a comparative study of our results with those of the American workers. We have, however, investigated a sample of fibrinolysin supplied by Loomis, and have shown that we can raise its activity appreciably by using a new method of fractionation, as described in the text. Although we did not attain a final purification of the enzyme, the facts described in the present paper may assist a further attack upon the problem.

EXPERIMENTAL

Preparation of material. Ox or human serum or plasma was obtained by prolonged centrifugation of fresh blood. After dilution with 10 vol. of distilled water and adjustment of pH to 5·3–5·5 with dilute acetic acid, the globulin precipitate, which settled overnight in the ice box, was collected by centrifugation and redissolved in one third of the original volume of 1% (w/v) NaCl (subsequently referred to as 'saline') and carefully neutralized with 0·1 N-NaOH. Chloroform (0·1 vol.) was added and the globulin solution was left for 3 or 4 days at room temperature with occasional shaking. The aqueous layer was then again diluted with distilled water (10 vol.), adjusted to pH 5·3, and the precipitate

allowed to settle in the ice box. It was centrifuged, redissolved in 0·2 of the original volume of saline and neutralized. This 'activated globulin solution' was used for further purification.

Fractionation with ammonium sulphate. Appropriate amounts of a saturated solution of $(NH_4)_2SO_4$ were added to the 'activated globulin solution'. After standing for 1 hr. at room temperature, some 'celite' was added and the precipitate filtered off. The filtrates were dialyzed (cellophan) against several changes of 1 % (w/v) solution of NaCl, neutralized and tested for fibrinolysis.

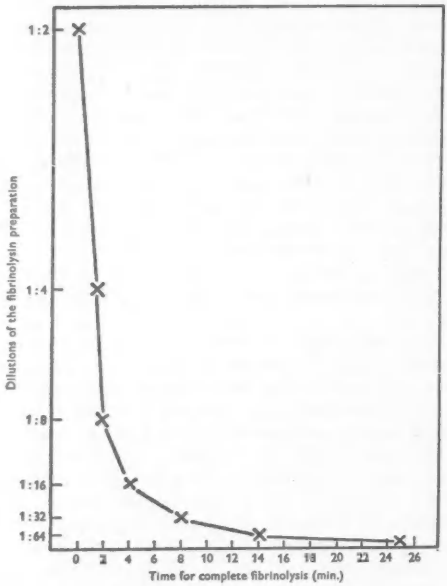

Fig. 1. Time-dilution curve of the activated ox-globulin preparation $OxGl_{10}$ (concentrated 3 times). From data corresponding to dilutions 1:8, 1:16 and 1:32, one can calculate the potency of the preparation as equal to 40 units/ml.; $U = 10c/t$, where $c = 1/D$, D being the dilution, and t the time required for complete fibrinolysis.

'Low-ionic' isoelectric precipitation. Isoelectric precipitation at low concentration of inorganic ions was performed, as described above, by diluting the activated globulin solution with distilled water (10 vol.) and adjusting to pH 5·0–5·5.

'Saline' isoelectric precipitation. The precipitate of the activated globulin solution was dissolved in 1 % (w/v) NaCl solution at pH 7·4, acetic acid added to bring the pH to 5·5–5·0 and the precipitate centrifuged and redissolved in neutral saline.

Delipidization of serum or plasma was performed according to the technique of McFarlane (1942) by adding ether (0·3 vol.), shaking and freezing to $-25°$ or $-30°$ with solid CO_2 (dry ice); after slow thawing at room temperature, the mixture was centrifuged and the clear bottom layer was carefully collected and diluted with 10 vol. of distilled water (pH 5·3) and the precipitate collected and treated in the usual way with 0·1 vol. of chloroform. As a rule, a duplicate sample of the original serum or plasma (non-delipidized) was treated in exactly the same way and the final activity of both samples compared.

Tyrosine was determined according to Folin & Ciocalteu (1927) in 0·1 or 0·2 ml. of an appropriate dilution of the material, N-NaOH (5 ml.), distilled water (4 ml.) and the phenol reagent (1 ml.) being successively added. After 20 min. standing, the colour was read in a Pulfrich spectrophotometer and the tyrosine concentration determined from a standard curve.

Cholesterol was estimated by the method of Bloor (1928), using the Pulfrich spectrophotometer for the final reading of the colour.

Fibrinolytic activity. The fibrinolytic tests were performed in small Wassermann tubes at 37°. To the solution of the enzyme to be tested (0·5 ml.), a 0·2 % (w/v) solution of human fibrinogen (0·3 ml.) and two drops of a thrombin solution (adjusted to produce clot in 1 min.) were added. The time for total lysis was measured and the number of units calculated. We found it convenient to define the unit as the amount of enzyme which produces total lysis in 10 min. under the conditions stipulated above (cf. Fig. 1).

RESULTS

Precipitation by ammonium sulphate

The whole of the active enzyme contained in the activated globulin fraction is precipitated between 25 and 37 % saturation (room temperature) with ammonium sulphate (Table 1). In a quantitative examination the precipitates at 0–40 % and 0–50 % saturation with ammonium sulphate were collected, redissolved in saline and dialyzed for 20 hr. The activity of the original solution was almost fully recovered.

Table 1. *Precipitation of fibrinolysin by different concentrations of ammonium sulphate at room temperature*

Materials added (ml.):						
Activated ox globulin	10	10	10	10	10	10
Ammonium sulphate (saturated solution)	—	5	6	7	8	10
Saline	10	5	4	3	2	—
Conc. ammonium sulphate (% satn.)	—	25	30	35	40	50
Lytic power of filtrates (units/ml.)	40	42	30	11	—	—

Influence of temperature on the activation of fibrinolysin after removal of the chloroform

The activity of the preparation was very seldom fully developed immediately after the chloroform treatment. Usually, the activity increased slowly, if the preparation was left a few days at room temperature. This spontaneous increase in activity was much slower if the material was kept in the ice box,

and very rapid if maintained at 38°. Figs. 2 and 3 illustrate such spontaneous increase in activity of the enzyme at 0°, at room temperature and at 38° respectively. The shape of the curve in Fig. 3 suggests an autocatalytic process, since the increase in activity is slow initially and rapid after 60–90 min.

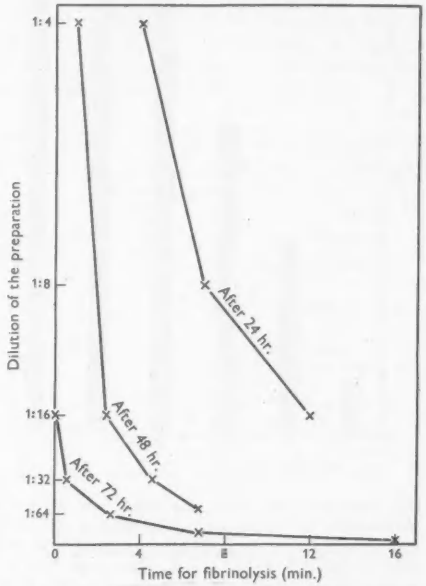

Fig. 2. Spontaneous activation at room temperature of preparation $OxGl_{11}$ after removal of chloroform.

After the maximum is attained, the activity of the enzyme slowly decreases again, possibly owing to the action of the products of the proteolysis. As a routine procedure, all preparations, after removal of the chloroform and precipitation at pH 5·3 with 10 vol. of distilled water, were maintained for 2–4 hr. at 38°. We have, however, observed that some of the preparations changed very little after incubation, probably because they were fully activated while in contact with the chloroform.

Christensen (1946) demonstrated that chloroform destroys the inhibitor normally present in plasma or serum. The shape of the curve of Fig. 3 disproves the idea that the further spontaneous activation might

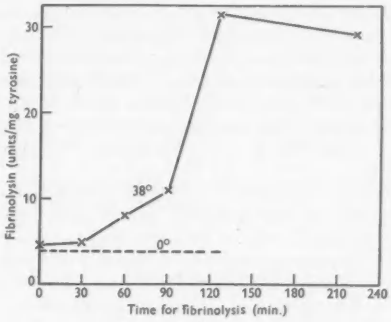

Fig. 3. Spontaneous activation at 0 and 38° of preparation $OxGl_{12}$ after removal of chloroform.

be due to a further destruction of the inhibitor, since in that case the maximal increase in activity would occur at the beginning of the incubation. That the enzyme is still present in the solution after the chloroform treatment, partially as an inactive precursor (pro-fibrinolysin) ready to undergo autocatalytic activation, seems to be the simplest conclusion to be drawn from our study of the effects of temperature upon the activation of the enzyme. All attempts to produce a spontaneous activation of the globulin solution without the chloroform treatment, even by incubating the original globulin solution for several hours at 38°, have failed. In a few experiments we have tried to activate the globulin solution with carbon tetrachloride and also with ether or ethanol, but in no case was any significant activation detectable by fibrinolysis tests.

Isoelectric fractionation in saline

Repeated low-ionic precipitation of an activated globulin (*AGl*) failed to raise its potency appreciably. However, by carrying out saline isoelectric pre-

Table 2. *Saline isoelectric fractionation of ox globulin preparation* $OxGl_{15}$

Conditions of experiments	Fibrinolysin (units/ml.)	Tyrosine (mg./ml.)	Fibrinolysin (units/mg. tyrosine)
I. Solution $OxGl_{15}$, after chloroform treatment	10	2·5	4
II. Above, after precipitation with 10 vol. distilled water (pH 5·5) and incubation at 38° (4 hr.)	65	2·2	30
III. Above, after isoelectric precipitation with 10 vol. distilled water ('low-ionic isoelectric precipitation')	85	1·7	47
IV. Above, after isoelectric precipitation in 1% (w/v) saline (three times) ('saline isoelectric precipitation')	24	0·07	343

cipitation, we obtained a rapid increase in activity/mg. tyrosine up to a maximum attained after three or four reprecipitations at pH 5·0–5·3.

Table 2 shows the increase in activity of the ox-globulin preparation $OxGl_{15}$, after chloroform treatment, incubation at 38° for 4 hr., reprecipitation at pH 5·5 with 10 vol. of distilled water and three precipitations in saline, at pH 5·1. A similar experiment with the preparation $OxGl_{16}$, in which incubation at 38° preceded low-ionic isolation precipitation, led to the same result, i.e. a marked increase in potency only when saline was substituted for distilled water.

In a few experiments we used human blood, defibrinated or kept citrated. In the latter case, no attempt was made to clot the plasma before the chloroform treatment, but at the end of this treatment the whole fibrinogen was found to be destroyed by the active enzyme. Table 3 shows a protocol of an experiment of isoelectric fractionation of human globulin solution (HGl_2) by three precipitations in saline at pH 5·3.

As seen from the examples given, repeated precipitations at pH 5·0 in saline raise the activity to a maximum which may be reached with two precipitations (cf. Fig. 4) or may require more. A limitation of this method of purification is that, when the initial activity of the enzyme was low, the maximum attained was also rather low. The only plausible explanation for this is that the inactive form of the enzyme (pro-fibrinolysin) is also precipitated in the same range of pH as the active form in such a way that further separation of the two substances by this type of precipitation becomes impossible. Table 4 records all our results with isoelectric precipitation in saline of different preparations of activated ox globulin and one human globulin preparation.

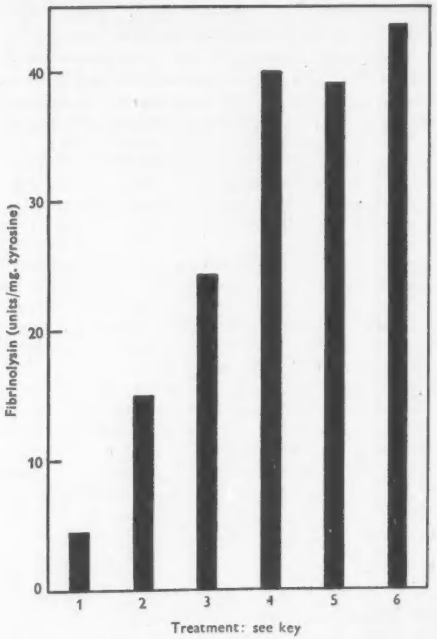

Fig. 4. Activation of preparation $OxGl_{18}$ and progressive purification by isoelectric precipitation in saline. Abscissae: 1, after chloroform treatment; 2, after incubation at 38° for 6 hr.; 3, after precipitation in saline at pH 5·0; 4, after repetition of 3; 5, after further repetition of 3; 6, after further repetition of 3.

It is quite clear from Table 4 that, if one starts with a very active preparation, purification by isoelectric precipitation in saline works very well, but

Table 3. *Human globulin solution (HGl_2) treated with chloroform and fractionated at* pH 5·0 *in saline*

Conditions of experiment	Fibrinolysin (units/ml.)	Tyrosine (mg./ml.)	Fibrinolysin (units/mg. tyrosine)
I. Activated human globulin	60	0·58	110
II. Above, after precipitation in saline at pH 5·0	50	0·25	200
III. Above, after reprecipitation in saline at pH 5·0	27	0·13	207

Table 4. *Increase in activity by fractionation at* pH 5·0–5·5 *of different globulin preparations*

Material	Activity (units/mg. tyrosine)	
	After chloroform and incubation at 38°	After isoelectric precipitation in saline (no. of reprecipitation in brackets)
$OxGl_{14}$	17	65 (4)
$OxGl_{15}$	65	343 (3)
$OxGl_{16}$ I	50	132 (3)
$OxGl_{16}$ II	24	158 (3)
$OxGl_{18}$ I	1	9 (4)
$OxGl_{18}$ II	15	48 (3)
Human Gl_2	110	207 (2)
Parke Davis preparation (Loomis *et al.* 1947)	114	300 (2)

when the initial activity is low, a less satisfactory result is obtained even after several isoelectric precipitations. That this method of purification is different from that recently described by Loomis *et al.* (1947) can be shown by the fact that fractionation in saline at the isoelectric point of a sample of one of the Loomis preparations, increased the specific activity three times. We feel, therefore, that purification by isoelectric precipitation in saline has a useful place, in combination with other methods, in the preparation of purified fibrinolysin.

in the usual way by isoelectric precipitation with 10 vol. of distilled water at pH 5·5. The precipitate collected by centrifugation was redissolved in saline, neutralized and submitted to the chloroform treatment, since there was no evidence that the simple treatment of delipidization was enough to activate the enzyme. Simultaneously, a sample of the original serum or plasma was submitted to identical treatment (delipidization excluded) and the final activities of both samples compared. The results (Table 5) show that a previous delipidization of the

Table 5. *Effect of a previous delipidization of serum or plasma by ether at −25° upon the activity of the enzyme resulting from subsequent chloroform treatment*

Exp.	Material used	Fibrinolysin (units/ml.)	Fibrinolysin (units/mg. tyrosine)	Cholesterol (mg./100 mg tyrosine)
I	$DOxGl_{21}$ (delipidized)	15	8·4	14·0
	$OxGl_{21}$	1	0·7	2·0
II	$DHGl_4$ (delipidized)	33	36·4	17·0
	HGl_4	20	25·0	9·0
III	$DHGl_5$ (delipidized)	55	55·0	12·5
	D_2HGl_5 (twice delipidized)	5	5·0	12·5
	HGl_5	9	10·0	3·7
	HGl_5E (ether saturated)	10	11·0	112·0
IV	$DHGl_3$ (delipidized)	40	88·8	8·3
	HGl_3	33	43·0	4·9
V	$DOxGl_{20}$ (delipidized)	12	14·0	28·0
	$OxGl_{20}$	0·5	0·5	15·0

Effect of delipidization of the original serum or plasma

The great variability of the results obtained after activation of the serum or plasma with chloroform induced us to study more thoroughly the mechanism by which chloroform activates the fibrinolysin precursor (pro-fibrinolysin). Christensen (1946) has shown that chloroform destroys the inhibitor, thus allowing spontaneous activation of the enzyme. It is, however, quite probable that the inhibitor, or at least one of the inhibitors, might be combined with the enzyme precursor. We have assumed as a working hypothesis that this linkage might be of a lipoprotein nature, and that chloroform might act by loosening such a linkage. We have, therefore, delipidized serum or plasma in an attempt to activate the enzyme, using first the technique of Hardy & Gardiner (1910) as modified by Hartley (1925). After treatment of the original serum or plasma with a mixture of ether and ethanol at −12°, the material obtained was entirely free from cholesterol or phospholipid, but had no fibrinolytic activity, nor could it be activated by chloroform. We, therefore, decided to try the milder delipidization described by McFarlane (1942), which consists of treating the plasma or serum with ether at −25° or −30°. After this treatment, the globulin fraction was prepared material definitely increased the fibrinolytic activity after chloroform treatment. In one of the samples of human plasma (D_2HGl_5), after complete thawing, the underlying plasma was submitted again to the cold-ether treatment with a definite decrease in the resulting activity. If one combines this result with that obtained by the Hardy & Gardiner (1910) method, it seems clear that a very mild initial delipidization will improve the final activity, but, if the process of delipidization is repeated, a permanent inactivation of the enzyme occurs.

From the data of Table 5, it might appear that a relationship exists between the final activity of the preparation and its cholesterol content. This seems to be true for most of the experiments in which the untreated material was compared with the delipidized one. In the experiment in which the plasma was saturated with ether (HGl_5E), without freezing, the amount of cholesterol present was, however, far greater than that in the delipidized fraction, whereas the final fibrinolytic activity was rather low. It is known that McFarlane's (1942) method does not remove all of the lipids present in serum or plasma. In our experiments 20–40 % of the cholesterol was not removed by this treatment. When the globulin fractions were prepared from delipidized serum or plasma, this residual cholesterol appeared to be concentrated in the globulin fraction; very frequently

Table 6. *Cholesterol contents of untreated and delipidized plasma or serum and their globulin fractions*

	Cholesterol content (mg./100 mg. of tyrosine)			
	Plasma or serum		Globulin fraction	
Material	Untreated	Delipidized	Untreated material	Delipidized material
Human P_4	31·0	5·0	9·0	17·0
OxS_{21}	8·7	3·1	2·0	14·0
OxS_{20}	18·0	6·0	15·0	28·0
Human P_3	—	—	4·9	8·3
Human P_5	34·0	7·0	3·7	12·5
DHP_5 (twice delipidized)	34·0	2·5	3·7	12·5

there was more cholesterol in the globulin fraction derived from a delipidized serum or plasma than in that from normal untreated plasma or serum (Table 6). This might be due to the fact that the material, from which the globulin fraction was precipitated, was saturated with ether. In favour of this possibility we might mention the result obtained with the ether-saturated material HGl_5E (Exp. III, Table 5) which contained much more cholesterol than any one of the other fractions obtained from the same human plasma. The results were, however, so paradoxical that a further study of this phenomenon is desirable. For the time being, however, we can accept the explanation that the presence of ether modifies the precipitability of a possible lipoglobulin material that might bear some relationship to the fibrinolytic enzyme.

SUMMARY

1. Experiments on activation and purification of the fibrinolytic enzyme of ox and human plasma or serum showed that the enzyme is associated with the globulin fraction precipitated between 25 and 37 % saturation with ammonium sulphate at room temperature.

2. After treatment with chloroform, the enzyme preparation can be further activated by incubation at 38° for a few hours. The activation process follows the course of an autocatalytic reaction.

3. 'Low-ionic isoelectric precipitation' (10 vol. of distilled water at pH 5·5) was compared with 'isoelectric fractionation in saline'. The former effected slight purification, whereas the latter rapidly enhanced enzymic potency, indicating that the enzyme is probably connected with a less soluble fraction of the total euglobulin fraction of plasma or serum.

4. A previous mild delipidization of plasma or serum led to a definitely higher activity of the enzyme after the chloroform treatment. A more complete delipidization appeared to destroy the proferment, since no activation could be obtained by subsequent treatment with chloroform.

5. Some relationship has been found between fibrinolytic activity and cholesterol content, especially in those fractions derived from mildly delipidized plasma or serum.

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A Study of the Behaviour of some Sixty Amino-acids and other Ninhydrin-reacting Substances on Phenol-'collidine' Filter-paper Chromatograms, with Notes as to the Occurrence of some of them in Biological Fluids

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Although the method of filter-paper chromatography (Consden, Gordon & Martin, 1944) can be applied to very many other types of compound (Partridge, 1946; Dent, 1947*a*; Vischer & Chargaff, 1947; Lugg & Overell, 1947; Maw, 1947), it is likely that its value will continue to be greatest in the amino-acid field. In this paper is presented a 'map of the spots' which may be of help to other workers. All the common amino-acids and many rare ones have been studied, also a few other interesting substances, some synthetic and not likely to be encountered in nature, which also give a ninhydrin colour reaction resembling that given by α-amino-acids. Some of the commoner 'unknowns' are also included since they appear to represent new compounds of wide distribution. To include all the 'unknowns' would be difficult, since it has been usual, when working with substances from natural sources, to stumble on them when least expected and in the most bewildering profusion.

EXPERIMENTAL

Points are only raised here which are additional to the earlier recorded work (Consden *et al.* 1944; Dent, 1946, 1947*a*).

Apparatus. The cabinet is shown in Fig. 1. It is convenient to use and construct. A cabinet of this type has given satisfactory service for two years of almost continuous use. It is much better to have two cabinets, one for the phenol and the other for the collidine runs.

Solvents. The phenol must be of A.R. or B.P. quality; too much decomposition with the formation of dirty brown material occurs if cruder phenol is used. An occasional batch of 'pure' phenol has been encountered which has decomposed unduly in spite of all possible precautions. It is a waste of time to persevere with such a solvent.

The nature of the collidine used has been of particular importance. Pure 2:4:6-collidine (2:4:6-trimethylpyridine) obtained from two different American sources completely failed to reproduce the results obtained in England by Consden *et al.* (1944) and by the writer, who all used the '2:4:6-' compound obtained from a British source. The R_F values were at least half of their reported ones, the insufficient separation of the amino-acids which thus ensued making the finished chromatograms almost worthless. On the other hand, a crude American 'collidine' believed to contain very little of the 2:4:6-isomer gave excellent results. By the kindness of the Reilly Tar and Chemical Corporation samples of pure 2:3-, 2:4- and 2:5-lutidines, of 2:4:6- and 2:3:6-collidines, of aldehyde-collidine (2-methyl-5-ethylpyridine) and of some crude boiling fractions of pyridine homologues were provided and tried out. The original British results could be well reproduced by taking a mixture of 1 vol. of

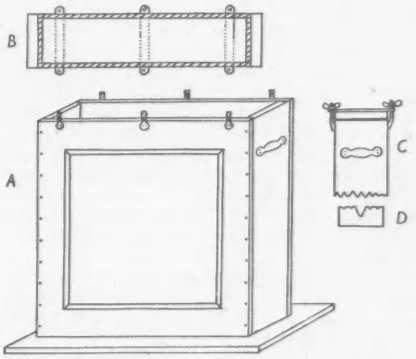


Fig. 1. Diagram of cabinet used in two-dimensional chromatography. The cabinet (*A*) is best made of $\frac{5}{8}$ in. thick 5-ply wood with the sides of one piece. The inside dimensions are 32 × 32 × 8 in. It is provided with a glass window 24 × 24 in. on each side. It must be screwed together firmly owing to a tendency to warp. The inside of the wood is heavily painted with paraffin wax. It is provided inside with a shelf (*D*) at both ends placed 4 in. from the top, the central notch takes the long glass trough, the lateral ones are used as alternative positions for the glass rods. The under-surface of the lid (*B*) has a rubber gasket which makes an airtight fit against the top edge of the cabinet when screwed down by the wing nuts as in *C*.

2:4-lutidine and of 1 vol. of 2:4:6-collidine and saturating it at the working temperature with 2 vol. of water. This mixture has been used here and is referred to in future as 'collidine'. This is not claimed to be the best possible mixture, and there is scope for further improvement to meet special points. When pure isomers are not available a crude fraction of pyridine bases boiling around 160° may be adequate. If the R_F values are too large and the spots ill-defined in consequence the fraction can be mixed with more

or less of the fraction boiling around 170°. There is some relation between the capacity of the solvent to take up water and its suitability for paper chromatography of amino-acids. When 5 ml. of water and 5 ml. of a good 'collidine' are mixed at about 23°, only about 1 ml. of the water remains undissolved. So far, when the water left over measures more than 1·5 ml. the solvent has given uselessly low R_F values.

General methods

A variation of more than 5° in temperature during a run is believed to be harmful, at least during the collidine run. The cabinet is therefore always kept, if possible, in an inside room without windows. The operating temperature is, however, less critical than was previously thought. Excellent results have been obtained at room temperatures of 12–31°.

The phenol was always used as the first solvent. Addition of about 0·1 g. of NaCN to one of the beakers in the bottom of the box was a great help in preventing oxidation of phenol. Just before closing the cabinet at the beginning of a run, four drops of ammonia solution (sp.gr. 0·880) were dropped in down the side. Four drops of diethylamine (Consden, Gordon & Martin, 1947) were likewise added just before a 'collidine' run. The sheets were dried at room temperature by directing an electric fan on to them overnight. The ninhydrin reaction was allowed to take place during 24 hr. at room temperature (24–30°). This avoids the danger of fading of the colour owing to overheating in the oven. With the exception of α-amino-*iso*butyric and of α-amino-α-methylbutyric acids (*q.v.*) all the amino-acids readily develop in this way. No difficulties with the 'pink front' of Consden *et al.* (1944) due to contamination by Cu salts have ever been encountered, even when no cyanide was added to the cabinet. Occasional batches of ninhydrin have been purchased that fail completely to give any colours with amino-acids.

The amino-acids were normally used in the form of a 0·01 M-solution, 5–10 μl. being nearly always adequate to give a good spot on the paper. The standard solutions are stable over many months if made up in 75% (v/v) ethanol, a small amount of conc. HCl being added to dissolve the less soluble ones. When reference is made to the appearance of this or that amino-acid in urine or blood it is to be assumed that volumes of 25 μl. of urine and of 125 μl. of protein-free plasma have been taken for the chromatogram (see Dent, 1946). There has been no systematic attempt to determine the lower limit of sensitivity for the detection of each amino-acid; the figures quoted are therefore usually on the high side. No mention is made as to the optical species, since there is good reason to believe that the active compounds and racemic mixtures travel similarly on the paper. The numerous substances which give very weak colours with ninhydrin are not included in the map, since under the conditions imposed, they are not likely to be present in large enough amounts to be confused with simple amines.

R_F values are not mentioned as such any longer. The rough figures can be surmised from Fig. 2 which is drawn to scale. The values in 'collidine' vary so widely with the isomer composition as to be worthless to record. Values in phenol are much more constant, but there is an occasional unaccountable variation of up to 15%, except under carefully controlled conditions, as when two sheets are run simultaneously in the same cabinet. It is stressed that the safest and simplest method of identification, now used exclusively, is from the pattern of the spots. This is remarkably constant and can be seen at a glance when many amino-acids are present together. When only a few are present the most convenient amino-acids to add as markers are glycine, alanine, valine and leucine. This extra step is not usually necessary because these same amino-acids are the ones most commonly present in greatest amounts in biological fluids.

Special methods

Effect of acidity during the phenol run. The basic amino-acids travel much more slowly under conditions of acidity which have little if any effect on the monoaminomonocarboxylic acids (Consden *et al.* 1944). This is, indeed, a very good test for basicity. The effect is even more marked with more basic substances such as glucosamine (*q.v.*). Perhaps the best way of carrying this out is to introduce during the phenol run a beaker of 50% (v/v) acetic acid into the cabinet, and, of course, to omit the ammonia. Equal volumes of conc. HCl and water may also be used in the beaker, but sometimes this mixture will also slow considerably the monoaminomonocarboxylic acids. The writer cannot confirm the claim of Consden *et al.* (1944) that the monoaminodicarboxylic acids travel quicker in phenol under acid conditions. Anomalies of their behaviour are discussed further under 'glutamic acid' and 'aspartic acid' in the section on results.

Hydrogen peroxide treatment. This continues to be a valuable technique for investigating the sulphur-containing amino-acids. The H_2O_2 is applied on the paper itself as previously described (Dent, 1947*a*), or about 5 μl. may be mixed beforehand with the sample. An interesting point, also discussed later, has arisen with regard to methionine sulphoxide. This substance does not oxidize further to the sulphone under the usual conditions which are adequate to oxidize methionine completely. Indeed, as far as can be judged from the chromatographic method, when a mixture of methionine and methionine sulphoxide is treated with H_2O_2 the methionine is converted to the sulphone, this presumably having to go through the sulphoxide stage of oxidation, while the sulphoxide which was present from the start remains largely unchanged. Addition, however, of 10–20 μl. of 0·02% ammonium molybdate to the spot on the paper allows the H_2O_2 to convert both methionine and its sulphoxide to the sulphone. There was always increased decomposition of the phenol after H_2O_2 had been used on the paper.

Routine method of investigating a potentially interesting fluid

The following routine is almost unavoidable with a new mixture containing many amino-acids, if all of them are to be detected and identified:

(1) Strip method (one-dimensional chromatogram) run with phenol. This serves as a rough qualitative and quantitative test. The volume of solution taken for the further tests may have to be adjusted on the basis of this result.

(2) Strip method with 'collidine'. Development is effected with the Pauly diazo reagent (Pauly, 1904) to show histidine and other diazo-reacting substances, which may not appear in the later tests owing to their relatively weak ninhydrin colour reactions.

(3) Paper square (two-dimensional) run with phenol and 'collidine'. This detects nearly all the amino-acids.

(4) Same as (3), but with prior H_2O_2 treatment. This detects cystine and methionine and will also affect other spots if due to dicovalent sulphur compounds (*q.v.*).

Further chromatograms may be necessary to cover specific points, especially after additions of pure amino-acids to act as markers.

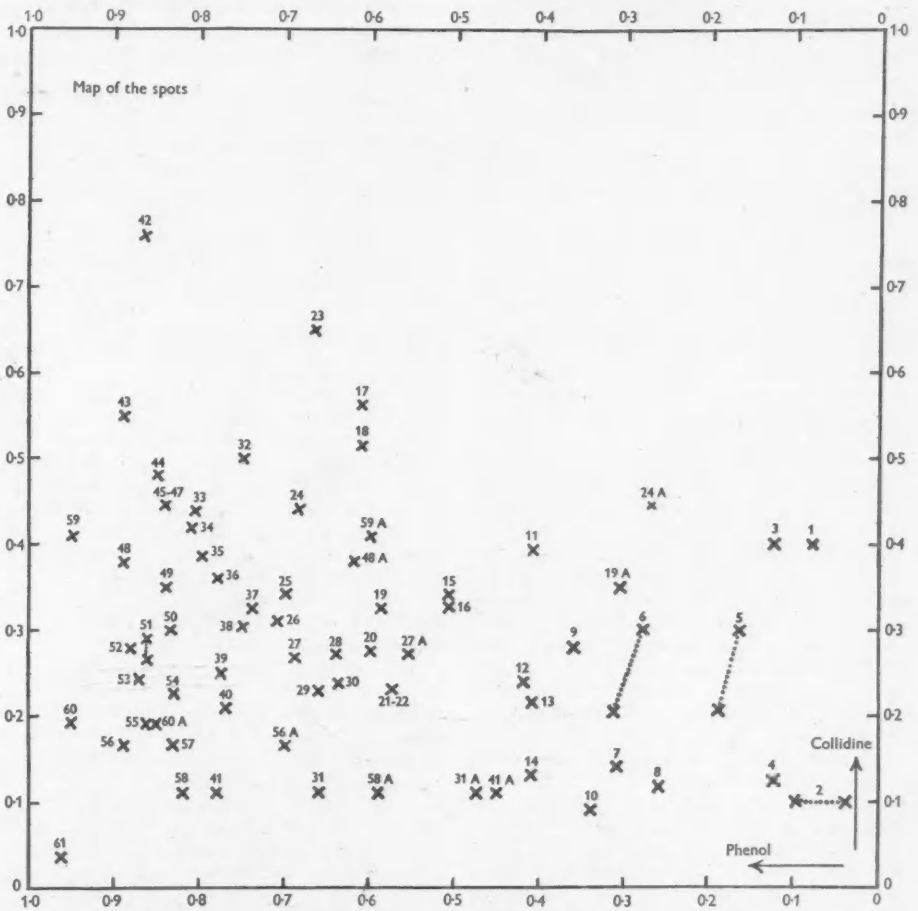


Fig. 2. The 'Map of the Spots'. The crosses show the average positions taken up on phenol-'collidine' two-dimensional chromatograms by the substances in question. See text for the identifications. The map is drawn to scale, assuming that the amino-acids start from the point at the right-hand bottom corner, and that the phenol is allowed to soak first along the paper from right to left, as far as the extreme left edge, then the collidine in an upward direction as far as the top edge. Approximate R_F values can be deduced from the scales at the edges. In practice, owing to the lower average R_F values in 'collidine', it is more convenient to run the 'collidine' for a greater distance on the paper than the phenol. This has the effect of spreading the spots upwards to a proportionate extent.

(5) Same as (3), but after hydrolysis with 6N-HCl for 24 hr. at 100°. The HCl must be removed by evaporation and the solution then made up to its original volume. Spots due to peptides, amides or tryptophan are removed by this process. Amino-acids liberated by the hydrolysis appear as new spots or as a strengthening of the spots already given by the unhydrolyzed fluid. It is important to remove traces of protein before the acid hydrolysis.

RESULTS

The behaviour of each amino-acid can be seen from the map (Fig. 2) and from the summaries below. Substances marked with an asterisk have only been run a few times on the chromatograms, and the results should be accepted with more reserve than

those from the other amino-acids, many of which have been run hundreds of times and under very variable conditions. The position of each substance on the map is shown by the number in parentheses after the name. The colour mentioned refers to the ninhydrin reaction and is to a minor extent dependent on the composition of the 'collidine' used.

Amino-acids or other known substances shown on the map

α-Alanine (20). Detectable in amounts of 2 μg. Gives a purple colour. Nearly always found in urine and other body fluids.

β-Alanine (29). Detectable in amounts of 5 μg. Gives a bluish purple colour. Its position on the map is so close to that occupied by citrulline that there is a serious possibility of confusion, indeed simple position matching has so far always failed to make distinction possible. See 'Citrulline' for a useful spot test. A substance giving a colour in this position is often found in blood, urine and in plant and tissue extracts. It has been proved to be β-alanine in some of these cases.

*alloThreonine (15). Detectable in amounts of 6 μg. Gives a purple colour. This unnatural amino-acid cannot be distinguished from threonine either in these solvents or in butanol.

α-Amino-n-butyric acid (26). Detectable in amounts of 4 μg. Gives a purple colour. This substance has been found in nearly every plant or animal tissue extract so far examined. It is usually present in much smaller amount than its neighbours, alanine and valine. It has not been found in acid hydrolysates of natural proteins, although it has been seen occasionally in the hydrolysates of chemically modified proteins, or in alkaline hydrolysates. Actual isolation in bulk for final confirmation of its structure remains to be done.

*α-Aminoisobutyric acid (37). Detectable in amounts of 4 μg. Gives a purple colour. The ninhydrin colour reaction occurs much less readily than with α-amino-*n*-butyric acid and the other common α-amino-acids. No colour develops after several days at room temperature. It soon appears, however, on heating to 100°. This is presumably due to a steric effect from the fully substituted α-carbon atom (see also α-Amino-α-methylbutyric acid). The unusual behaviour with ninhydrin allows of an easy distinction from the isomeric *n*-compound. This acid has not yet been found in nature.

γ-Aminobutyric acid (40). Detectable in amounts of 5 μg. Gives a purple colour. A substance has been found in traces in blood and urine which matches its movements exactly. Both are stable to H_2O_2, to prolonged acid hydrolysis, and are not affected by running the chromatogram at an acid pH during the phenol run. It is probably one of the substances found in fair quantity by Dent, Stepka & Steward (1947) in potato extracts (no. 23 in their photograph). In the absence of close neighbours to act as reference points it may be confused with the commonly occurring methionine sulphoxide. In such a case, its identity may be confirmed by adding markers or by its stability to H_2O_2 in the presence of ammonium molybdate.

*ε-Aminohexanoic acid (55) (ε-amino-*n*-caproic acid). Detectable in amounts of 5 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the α-amino-acids. Its position is very different from that occupied by its isomer, norleucine. The acid is not believed to occur in nature.

*α-Amino-ε-hydroxycaproic acid (38). Detectable in amounts of 10 μg. Gives a purple colour. Not believed to occur in nature. A spot in this exact position has, however, been given by the acid hydrolysate of deaminized casein, i.e. casein treated with HNO_2. In the same hydrolysate only a trace of lysine was found.

*α-Aminooctanoic acid (43) (α-aminocaprylic acid). Detectable in amounts of 6 μg. Gives a purple colour. It has not yet been found in nature.

*δ-Aminopentanoic acid (57) (δ-amino-*n*-valeric acid). Dectectable in amounts of 5 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the α-amino-acids. Its position is very different from that occupied by its isomer norvaline. The acid is not believed to occur in nature.

*α-Aminophenylacetic acid (33). Detectable in amounts of 8 μg. Gives a purple colour. It is not believed to occur in nature.

Arginine (56). Detectable in amounts of 15 μg. Gives a purple colour. In the presence of 1 : 1 (v/v) acetic acid it takes up the position 56A. The rate of movement in phenol is slowed still further in the presence of 5N-HCl. Its presence may be confirmed by means of the Sakaguchi reaction directly applied to the spot on the paper (Consden, Gordon & Martin, 1946*a*). However, this reaction is of limited value since it is less sensitive than the ninhydrin test and does not work well on spots previously developed with ninhydrin. Arginine is very commonly found in biological fluids.

Asparagine (13). Detectable in amounts of 5 μg. Gives an orange brown colour, sometimes tinged with olive. Although it moves very close to glycine, overlapping always taking place when both are present, it can usually be distinguished by the difference in the colour. Presumably a small amount of asparagine could not be detected in the presence of a large amount of glycine, since the large spot then given by the latter would completely overlap the former. Asparagine has moved rarely to a position slightly above glycine. It has been found in pathological urine, and, of course, in large amounts in plant extracts and also in Amigen—a commercial brand of enzymic casein hydrolysate. It must not be

confused with an unidentified substance, stable to hydrolysis, which also gives a yellow spot and is present in biological extracts. This substance (not shown on the map) moves to a position just below asparagine.

Aspartic acid (5). Detectable in amounts of 5 μg. Gives a bluish purple colour which develops a little more slowly at room temperature than do the colours of the other common amino-acids. The spot usually shows streaking in the direction of the line shown, or may even appear as two distinct spots one over the other. The reason for this is not yet known. The acid is not usually found in normal urine, but often in pathological ones with a high concentration of amino-acids (Dent, 1947*b*). When found in natural sources the spot is often distorted by an unidentified yellow substance which moves between aspartic and glutamic acids.

Urines which do not contain detectable aspartic acid or asparagine often contain large amounts of the former after acid hydrolysis. Presumably the aspartic acid is present in the original urine as a simple derivative, such as an acyl one.

**Carnosine* (54). Detectable in amounts of 15 μg. Gives a brownish yellow colour which turns greenish if heated to 105°. It has not yet been found in blood or urine.

Citrulline (30). Detectable in amounts of 5 μg. Gives a reddish purple colour. It may be confused with β-alanine which moves to a similar position. It can be distinguished from β-alanine by treating the spot on the paper with 5% (w/v) *p*-dimethylaminobenzaldehyde in N-HCl with which it gives a bright yellow colour, in common with urea and many other urea derivatives, whereas β-alanine does not give a colour reaction. Its previous treatment with ninhydrin does not interfere. The aldehyde reaction for citrulline is less sensitive than the ninhydrin colour reaction, hence a negative test must only be considered significant when the ninhydrin reaction is very strong. A weak spot in the position of citrulline and giving the typical reddish purple ninhydrin colour is often seen in normal blood and urine. However, the presence of citrulline in these fluids has not yet been confirmed by the spot test.

Cystathionine (7). Detectable in amounts of 8 μg. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and decomposes during the collidine run. It has not yet been found in nature by these methods.

Cysteic acid (1). Detectable in amounts of 5 μg. Gives a blue colour. It is formed, apparently quantitatively, from cystine, by treatment with H_2O_2. Old solutions of cystine may show the presence of cysteic acid. Traces of the latter, occasionally found in urine, may possibly have arisen from cystine by oxidation during storage. Urine from a patient with cystinuria, when treated with H_2O_2, contains easily detectable amounts of cysteic acid. This has occurred with the fresh urine as well as with urine that has stood until cystine crystals have deposited. No further evidence has therefore been secured in favour of the theory that the cystine is originally secreted in the form of a complex which decomposes on standing (Brand, Harris & Biloon, 1930). Cysteic acid is found in the hydrolysate of casein treated with H_2O_2 according to the method of Toennies (1942), and also in the hydrolysate of aged wool (Consden *et al.* 1946*b*).

Cysteine and cystine. These compounds are not usually seen in the chromatograms, at least when present in amounts of a few μg. If large quantities are used, traces of cysteic acid may be detected and also spots due presumably to partial oxidation products. Consden *et al.* (1944), however, report a position for cystine on their chromatograms. The only apparent difference in their working conditions is in the use of the solvents in the reverse order, i.e. 'collidine' first, then phenol.

Diiodotyrosine (17). Detectable in amounts of 40 μg. Gives a dull greenish purple, slightly duller and browner than that given by tyrosine. It readily couples with diazotized sulphanilic acid in Na_2CO_3 solution (Pauly reagent) to give a brownish red azo dye, presumably with the elimination of iodine. Previous development with ninhydrin does not interfere with the diazo reaction.

**ββ-Dimethylcysteine* (penicillamine). It decomposes on the chromatogram when applied in amounts of a few μg. Some $\beta\beta\beta'\beta'$-tetramethylcystine may be formed, however, and is then picked up in its accustomed position (*q.v.*).

**Djenkolic acid* (14). Detectable in amounts of 5 μg. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run.

Ethanolamine (48). Detectable in amounts of 5μg. Gives a purple colour. A serious loss of the material by volatilization always occurs if the paper is dried at 100°, and apparently to some extent when all the operations are carried out at room temperature. When the phenol run is carried out in the presence of 5N-HCl the substance finally occupies the position 48A. It is believed that ethanolamine is the substance in pathological urines, previously reported under the name 'fast-valine' (Dent, 1947*b*). It has also been found in the hydrolysate of gramicidin (Synge, 1945).

Ethanolamine-phosphoric acid (10)

$$(NH_2CH_2CH_2.O.PO(OH)_2).$$

Detectable in amounts of 10 μg. Gives a purple colour; the colour produced at room temperature usually intensifies on heating. It is remarkably stable to acid hydrolysis, plenty of the material

surviving 6N-HCl for 24 hr. at 100°. It is unaffected by H_2O_2. A substance has been found in cancer tissue, intestinal mucosa and body fluids which matches the movement exactly on the chromatogram and also shows the above properties. Ethanolamine-phosphoric acid has been isolated from intestinal extracts by Colowick & Cori (1939).

Glucosamine (24). Detectable in amounts of 16 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the common α-amino-acids. When the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid it takes up the final position 24A. Glucosamine has been frequently found in the hydrolysates of naturally occurring proteins and is presumably derived from combined polysaccharide.

Glutamic acid (6). Detectable in amounts of 3 μg. Gives a purple colour. It often occurs as a streak along the direction of the dotted line, the bulk of the colour sometimes being collected at the two ends, almost as if it were trying to form two separate spots (compare the behaviour of aspartic acid). The lower part is always much redder in colour. At other times it may take up an even lower position on the map, thus causing serious danger of confusion with the other amino-acids in that region. This very low position is well illustrated in the photograph published by Martin (1947) of the chromatogram given by hydrolyzed gelatine. No reason can be suggested for these unpredictable anomalies of behaviour. Further difficulties suggestive of peptide formation arise when old solutions of glutamic acid are analyzed on the chromatograms. A two year old solution of glutamic acid in 0·4N-HCl has appreciable quantities of another ninhydrin-reacting substance which moves much more slowly than glutamic acid in phenol and is decomposed back into the latter on acid hydrolysis. Equally old solutions in 75% (v/v) ethanol have given up to four spots widely separated on the paper. The main one of these, which travels to a position between leucine and ethanolamine, is detectable when the solution is only a few days old, and appears to be the γ-ethyl ester. Together with other anomalies in the behaviour of glutamic acid already reported in the literature these findings suggest that some fundamental facts in the simple chemistry of the compound still await discovery. Traces of glutamic acid are usually found in blood, urine, and plant tissues. Large amounts may be further liberated by mild acid hydrolysis even when only mere traces of glutamine are present. Presumably the glutamic acid in such cases is present in the form of a simple *N*-derivative, such as an acyl one (compare aspartic acid).

Glutamine (21). Detectable in amounts of 5 μg. Gives a purple colour. Incorrectly stated to move to the same position as alanine (Dent, 1947*a*), this substance moves to a position indistinguishable from that believed also to be occupied by a peptide (see 'Under-alanine') containing serine and glycine. Owing to this behaviour the identification of glutamine must always be confirmed by hydrolysis, which should result in the disappearance of the glutamine with simultaneous appearance of at least a corresponding amount of glutamic acid but no increase in the serine or glycine. With this method it now appears that the substance found in this position in normal blood and urine is glutamine and not the peptide 'under-alanine' as previously claimed by the author (Dent, 1947*a*). Glutamine has also been detected in large amounts in pathological urines, in plant extracts and in Amigen.

**Glutathione* (4). Detectable in amounts of 10 μg. Gives a purple colour. A spot in this position has been given by tissue extracts. The position given by pure glutathione has not, however, been very exactly reproducible. Further work is required to determine the optimum working conditions for its identification.

Glycine (12). Detectable in amounts of 1 μg. Gives a slightly reddish purple colour. It is usually the main amino-acid in urine, but is usually found in any biological fluid. After its hydrolysis by acid, urine often shows a large increase in glycine, presumably obtained from hippuric acid.

Histamine (59). Detectable in amounts of 16 μg. Gives a dull greenish purple colour. Its identity can be confirmed by the bright red colour reaction which it gives with the Pauly reagent. The vapour from 1 : 1 (v/v) acetic acid does not affect its movement in phenol. If 5N-HCl be present, however, it is greatly slowed and ends up in the position 59A. It has been found in tissue extracts and possibly in pathological urines. There is a serious danger of losing the histamine owing to its volatility.

Histidine (27). Detectable in amounts of 20 μg. Gives a dull greenish purple colour. The strength of colour seems to be definitely less than that given by an equimolecular amount of the common α-amino-acids. Hence the presence of histidine is better detected by means of the Pauly reagent with which it gives an intense red colour. For this test the paper is freed from all traces of phenol or 'collidine' by leaving it overnight in the oven at 100° or by thorough washing with benzene or acetone. Previous use of ninhydrin does not interfere with the Pauly reaction. The position of histidine on the chromatogram varies appreciably with the nature of the 'collidine' used. Pure 2:4:6-collidine brings it to a position well below the level of methionine sulphoxide (no. 39 on the map), and also results in a much browner ninhydrin colour. Some impure 'collidines' of unknown isomer composition may raise it almost to the level of α-amino-*n*-butyric acid (no. 26). The movement in phenol is not slowed by the vapour of 1 : 1 (v/v) acetic acid. It is, however,

slowed to position 27A in the presence of 5N-HCl. Commonly found in biological fluids.

**Homocysteic acid* (3). Detectable in amounts of 10 μg. Gives a blue colour. It is readily formed from homocystine by the action of H_2O_2. It may be confused with cysteic acid unless careful matching is done. Not yet found in nature.

**Homocystine*. This substance does not appear on the chromatogram owing to its decomposition.

Hydroxylysine (31). Detectable in amounts of 15 μg. Gives a dull purple colour. Its position on the chromatogram agrees with the rule that the addition of a hydroxyl group on to the β-carbon atom of an amino-acid (lysine in this case) lowers the R_F in phenol with little effect on the R_F in collidine. The amino-acid moves to position 31A when the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid. It has not yet been found chromatographically in natural sources, although the photograph of the chromatogram from hydrolyzed gelatine published by Martin (1947) shows a suspicious smudge in the appropriate position.

Hydroxyproline (28). Detectable in amounts of 15 μg. Gives a brownish yellow colour. The spot usually touches that of alanine, but the two are easily distinguished by colour. Found a few times in urine, more often in hydrolyzed urines.

**Lanthionine* (8). Detectable in amounts of 6 μg. Gives a purple colour. Treatment with H_2O_2 produces a compound which travels more slowly in phenol and which decomposes during the collidine run. Not yet found in nature.

Leucine (45). Detectable in amounts of 10 μg. Gives a purple colour. Coincides with isoleucine and norleucine in the phenol-'collidine' chromatogram. Identification of a spot in this position is therefore best done after isolating the pure amino-acid by the cutting-out technique (Dent, 1947*a*; Consden *et al.* 1947). The material must then be run on a strip of paper (one-dimensional chromatogram) with butanol as solvent. The isomers are readily separated from each other in this way and should be identified by simultaneous comparison with standards of the pure amino-acids. Norleucine travels the fastest in butanol and is followed by leucine and then by isoleucine. Alternatively the original chromatogram could be run again with butanol as first solvent in the place of phenol. The positions with this solvent combination have not yet, however, been worked out accurately enough. In the absence of unidentified amino-acids whose behaviour in the solvents is unknown, the three leucines can be distinguished by a one-dimensional chromatogram run with benzyl alcohol or butanol on the original mixture (Consden, Gordon, Martin, Rosenheim & Synge, 1945). Leucine is commonly found in biological fluids.

Isoleucine (46). Detectable in amounts of 10 μg. Gives a purple colour. See 'Leucine' for identification. It is commonly found in biological fluids.

Lysine (58). Detectable in amounts of 15 μg. Gives a dull purple colour. Its spot always tends to be larger than the others and often shows streaking to the right. It takes up position 58A when the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid. In the presence of 5N-HCl much greater slowing occurs in the phenol. It usually overlaps ornithine if present. For their identification in the presence of each other see 'Ornithine'. Commonly found in biological fluids.

Methionine (34). Detectable in amounts of 10 μg. Gives a purple colour. Overlaps with leucine, although it may be distinguished when present in high concentration. Its presence is best confirmed by the H_2O_2 technique (*q.v.*). Methionine slowly oxidizes to the sulphoxide if left to stand some days on the filter paper. It oxidizes more readily during the development of the chromatogram so that a pure sample of methionine usually gives two spots of about equal strength, one for the methionine and the other for its sulphoxide. This artifact of oxidation occurs mainly during the phenol run. The presence of methionine in the original sample may therefore be suspected whenever the sulphoxide is found in its characteristic position (no. 39) (see below for further details). The platinum reagent described by Consden *et al.* (1946*a*) can also be used to detect methionine. The test, however, is less sensitive than the ninhydrin colour reaction and cannot be applied after ninhydrin. It is not positive for the sulphoxide or sulphone, or for cystine. Methionine is only very rarely detectable in biological fluids.

Methionine sulphone (25). Detectable in amounts of 10 μg. Gives a purple colour. Is formed, apparently quantitatively, by treating methionine with an excess of H_2O_2. Not believed to occur in biological fluids. It has been found in the hydrolysate of casein treated with H_2O_2 by the method of Toennies (1942).

Methionine sulphoxide (39). Detectable in amounts of 5 μg. Gives a purple colour. Treatment with H_2O_2 has hardly any effect on it, at most only a trace being further oxidized to the sulphone. In the presence of ammonium molybdate, however, H_2O_2 converts it quantitatively to sulphone. This serves as a most specific confirmatory test of its identity. It is only rarely found in biological fluids. When methionine sulphoxide is found in biological fluids, the question whether it was present as such in the original fluid or was formed from methionine during the phenol run can be settled by treatment with H_2O_2 without a catalyst. The sulphoxide, if present, survives the oxidation and is again found on the chromatogram. If only methionine is present it will all be oxidized to sulphone, and no sulphoxide is found. This

method has been used to confirm the excretion of sulphoxide, as such, in the urine, after giving methionine by mouth (Dent, 1947*a*).

**α-Methyl-α-amino-n-butyric acid* (49). Detectable in amounts of 10 μg. Gives a purple colour. As with α-amino*iso*butyric acid which is also fully substituted at the α-carbon, the colour does not develop on standing a few days at room temperature. It slowly appears on heating to 100°. This behaviour enables it to be easily distinguished from neighbouring substances on the chromatogram. It is believed not to occur in nature.

Methylhistidine (51) (obtained from anserine). Detectable in amounts of 20 μg. Gives a dull greenish purple colour closely matching that given by histidine. It does not give any reaction with the Pauly reagent. Its spot partly overlaps that of proline from which it is easily distinguished by its colour. As shown on the map its relation to proline (no. 52) in a vertical direction is subject to a little variation. The position slightly above proline is more usual. A substance assuming exactly its position and matching its colour with ninhydrin has been found in several pathological urines and has appeared as the strongest spot in the chromatogram from a normal dog's urine.

**Monoiodotyrosine* (23). Detectable in amounts of 30 μg. Gives a dull greenish purple colour, closely matching that given by tyrosine. It readily reacts with the Pauly reagent to give a brownish red compound indistinguishable from that given by tyrosine. Its position is very close to, if not identical with, one of the radioactive spots found by Fink, Dent & Fink (1947) in the chromatogram of the hydrolysate of a rat's thyroid gland removed 24–48 hr. after an injection of ^{131}I; otherwise no evidence has been obtained of its possible occurrence in nature.

**Norleucine* (47). Detectable in amounts of 10 μg. Gives a purple colour. See 'Leucine' for method of identification. Not believed to occur in biological fluids.

**Norvaline* (35) (α-amino-*n*-valeric acid, α-aminopentanoic acid). Detectable in amounts of 5 μg. Gives a purple colour. Not clearly distinguishable from valine, although a spot made up of both valine and norvaline may appear to be double. To distinguish between the two the substance giving the spot must be isolated by the cutting out technique (Dent, 1947*a*; Consden *et al.* 1947) and the substance run on a paper strip (one-dimensional chromatogram) with butanol. This readily separates them, the straight chain compound as usual travelling faster than its branched isomer. It is not believed to occur in nature.

Ornithine (41). Detectable in amounts of 10μg. Gives a purple colour. The spot always overlaps that given by lysine if both substances are present together. The two substances appear at first sight to be giving one large spot. The slightly different shades of the colour reaction can, however, usually be observed. For final confirmation of identity the chromatogram must be repeated with the vapour of 1 : 1 (v/v) acetic acid present during the phenol run. Under these circumstances the much greater slowing of the ornithine (to position 41A) compared with the lysine causes two distinct spots to be produced which are then easily identified. Ornithine has not yet been found in blood and urine.

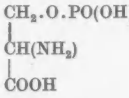
Penicillamine. See *ββ'*-dimethylcysteine.

Phenylalanine (44). Detectable in amounts of 10 μg. Gives a greenish purple colour; with other 'collidines' the colour may be blue or even brownish. Addition of a trace of diethylamine to the cabinet is necessary for the phenylalanine to move to the position shown (Consden *et al.* 1947). In the absence of diethylamine or if only ammonia is added it invariably moves slower in the collidine and then overlaps the leucines seriously. It is often found in pathological urines.

Proline (52). Detectable in amounts of 8 μg. Gives a lemon yellow colour. It is often found in pathological urines.

Serine (9). Detectable in amounts of 2 μg. Gives a purple colour which may appear brownish when impure 'collidine' is used. It is often found in traces in normal urine, much larger amounts may be excreted in disease (Dent, 1947*b*).

**Serine-phosphoric acid* (2).



This was examined in the form of the Ba salt and was detectable in amounts of 10 μg. Gives a purple colour. It has given either a single or a double spot under apparently similar conditions. There is some evidence, however, that the part of the spot moving faster in phenol occurs when the concentration of ammonia is slightly greater. It has not yet been found free in nature. It is present in a combined state in casein and vitellinic acid.

Taurine (11). Detectable in amounts of 3 μg. Gives a purple colour. The ninhydrin reaction occurs as readily as with the common α-amino-acids. Owing to its characteristic position this is a most useful substance to add as a 'marker' to help in the identification of neighbouring amino-acids. Its position is not altered by the presence of acetic or hydrochloric acids during the phenol run. It is commonly found in normal blood and urine. In pathological urines it may be excreted in very large amounts to become the strongest spot on the chromatogram. This substance was alluded to under the name 'over-glycine' (Dent, 1947*a*) before it was identified. Paper chromatography probably pro-

vides the only simple specific test for this widely distributed substance. Many of the methods for determination of amino-acids in the urine will include taurine as contributing to the 'total amino-nitrogen'. Nitrous acid and formol methods will do so, and will therefore give quite incorrect results in some pathological urines containing an excess of taurine. The copper method (Albanese, Frankston & Irby, 1944) and the ninhydrin-CO_2 method (Van Slyke, MacFadyen & Hamilton, 1943) do not determine taurine.

$\beta\beta\beta'\beta'$-Tetramethylcystine (19) (oxidized penicillamine). Detectable in amounts of 10 μg. Gives a purple colour. Treatment with H_2O_2 and ammonium molybdate causes some of it to be oxidized to an unidentified substance which gives a typical ninhydrin reaction and moves to position 19A. It has not yet been found in biological fluids.

Threonine (16). Detectable in amounts of 10 μg. Gives a purple colour. The colour strength given by this amino-acid appears to be weaker than that given by the commoner α-amino-acids. The vertical position varies slightly with the nature of the 'collidine' used. With some crude batches it has been moved down nearly to the level of alanine. It is commonly found in biological fluids.

Thyroxine (42). Detectable in amounts of 60 μg. Gives an olive brown colour going greener on heating. Thyroxine moves so fast in the collidine that it may run into a brown impurity which usually shows on the finished paper in this region. This impurity is always formed during the phenol run and is subsequently carried forward in the collidine a little way behind its leading edge. When it does this, the thyroxine may be completely lost. There is clearly room for the development of a specific test for this substance which is more sensitive than the ninhydrin reaction, and which gives a colour which will show up against the occasional brown background. So far no success has been obtained in applying the nitrous acid reaction of Kendall & Osterberg (1919).

Tryptophan (32). Detectable in amounts of 20 μg. Gives a purple colour. It is not usually present in blood and urine, although it has been found in the urine of patients with severe liver damage (Dent, 1947*b*). Not found in acid hydrolysates of proteins owing to its destruction by the acid.

Tyrosine (18). Detectable in amounts of 15 μg. Gives a dull greenish purple colour. Because of the very characteristic and unambiguous position of its spot, tyrosine is a most useful 'marker'. It gives, in similar strength, a brown colour with the Pauly reagent. A sample of 'pure L-tyrosine' from a reputable manufacturer was found to contain a large quantity of leucine. It is commonly found in biological fluids.

'*Under-alanine*' (22). This name was given by Dent (1947*a*) to a substance present in the urine of a patient with Fanconi syndrome and cirrhosis of the liver. The substance gave a reddish purple colour with ninhydrin and, on hydrolysis of the urine, the subsequent chromatogram contained no 'under-alanine' and showed a remarkable increase in the content of serine. Experiments on the isolated substance suggested that it might be the tripeptide serylglycylglycine. This substance has not been encountered again, although there is no reason to doubt the original finding. It is now known that both glutamine and a substance in normal blood and urine and in some other pathological urines also move to a very similar position to that of the tripeptide. It is now considered that the substance in normal blood and urine is glutamine and not the tripeptide, as claimed by the writer (Dent, 1947*a*). It is considered safe to say that a spot in this position is due to glutamine if it disappears on hydrolysis to liberate at least an equivalent amount of glutamic acid without increasing the serine content perceptibly.

Valine (36). Detectable in amounts of 3 μg. Gives a purple colour. Nearly always found in normal and pathological blood and urine and in tissue extracts of plants and animals.

Commonly occurring unidentified substances

'*Fast-aminobutyric acid*' (50). This is an unidentified substance which has been frequently found in plant extracts and in blood and urine. It is the substance numbered 24 in the photograph of the chromatogram of the potato extract published by Dent *et al.* (1947). It is stable to acid hydrolysis and to H_2O_2 and is not slowed during the phenol run by working at an acid pH. It is therefore believed to be a monoaminomonocarboxylic acid. It does not react with diazotized sulphanilic acid or with *p*-dimethylaminobenzaldehyde.

'*Fast-arginine*' (60). This is an unknown substance giving a typical purple colour with ninhydrin. It is stable to acid hydrolysis, and appeared on one occasion to concentrate in a fraction precipitated with phosphotungstic acid. When the phenol run is carried out in the presence of the vapour from 1 : 1 (v/v) acetic acid it is slowed to the position 60A. It is therefore believed to be a new basic amino-acid. It has been found several times in blood and tissue extracts and was one of the main amino-acids to be passed in the urine of a patient with Fanconi syndrome. Its movement on the chromatogram suggests that it may be the guanidine compound derived from lysine, i.e.

$NH_2.C(:NH).NH.CH_2.CH_2.CH_2CH_2.CH(NH_2).COOH$.

'*Green-spot*' (53). An unidentified substance giving a bright green colour with ninhydrin. It has been seen in many normal and pathological urines. It survives hydrolysis with 6N-HCl for 24 hr. at 100°.

'*Nephrosis-peptide*' (61). An unidentified substance found in large amounts in the deproteinized urine in one out of three patients with nephrosis. It was found in the blood of one of the patients who was not passing it in the urine. It gives a typical purple colour with ninhydrin, and on hydrolyzing liberates most of the common amino-acids. Traces of a substance moving to a similar position have occasionally been seen in other urines.

Amino-acids not shown on the map

Thiolhistidine gives bands in phenol of R_F 0·22. In collidine, however, much decomposition appeared to take place, for the substance which gave a very weak ninhydrin colour at R_F 0·42 no longer gave the Pauly reaction.

Dihydroxyphenylalanine gave bands in phenol and collidine (R_F 0·33 and 0·44 respectively) but has never been seen in a two-dimensional chromatogram. Presumably it decomposes during the longer time necessary for this process.

Peptides not shown on the map

Anserine moves readily on the chromatogram to a position near proline. The ninhydrin reaction, however, is so weak that it is unlikely to be picked up unless present in very large amount. Anserine (75 μg.) has been run on a two-dimensional chromatogram without being subsequently found. It is more easily seen on one-dimensionals. Under such circumstances it gives a dull greenish colour with ninhydrin.

Purely synthetic peptides do not come within the scope of this paper.

Substances that do not react with ninhydrin

Pyrrolidone carboxylic acid does not give a ninhydrin reaction and its movement on the chromatogram has not yet been traced.

This is no place to mention in detail substances which do not react with ninhydrin. It is, however, repeated that the previous suggestion of the writer (Dent, 1947*a*) that the paper chromatographic method might be of very general application to organic and inorganic substances has been fully confirmed in subsequent trials. The easy separation of chlorides, bromides and iodides by collidine is quoted as a further example of the latter. As a result of this it is useful to bear in mind that the same paper chromatogram which has been run on, say, a specimen of urine, can often, after the amino-acids have been identified by development with ninhydrin, be again used for the identification of other substances present, e.g. urea, which moves to a position below tyrosine on the map. There is very great scope therefore for the extension of the method to simultaneous qualitative analysis covering a wide range of compounds likely to be present in biological fluids.

DISCUSSION

The chief point relative to the more general use of the paper chromatographic method concerns the question of specificity. How far is one justified in assuming that a spot in the exact position of, say, alanine, is in fact due to alanine?

First must be considered the question of the specificity of the ninhydrin colour reaction. There is still much confusion on this subject. For instance, Schmidt (1944) still states erroneously that β- and γ-amino-acids do not give the reaction. As shown here, where only reactions leading to good colour strength are referred to, many primary amines, as well as the β-, γ-, δ-, and ϵ-amino-acids will give typical reactions, the rate of development of the colour, the strength and the actual shade being identical with that given by α-amino-acids. However, with mixtures obtained from natural sources only a limited number of such substances is likely to be present. The simple amines are readily detected by subsequent tests depending on the great difference in rate of movement in phenol caused by a difference in acidity (e.g. glucosamine). Substances like taurine may be confused with amino-acids, although it seems likely that sulphonic acids will always be detectable by their unusually fast movement in the 'collidine'. The δ- and ϵ-amino-acids have so far only been reported to be formed as the result of putrefaction (Neuberg, 1911 and Ackermann, 1910, respectively).

Secondly, the question of the accuracy of the position matching arises. Here it must be repeated that there is no need to determine R_F values when enough different amino-acids are present to enable identification from the pattern of the chromatogram. With very few exceptions the latter is quite extraordinarily constant, since day to day variables always and variations in the isomer composition of the collidine nearly always affect the R_F values in the same proportions and do not therefore alter the pattern. When a spot giving the typical ninhydrin reaction occurs in the pattern in the exact position of alanine, then the probability is overwhelming that the unknown substance is in fact alanine. A final check must still be done, however, by adding pure alanine to the mixture and then observing that under these identical conditions the pure substance runs exactly with the unknown, and that it is quite stable to hydrolysis. Hydrolysis is most essential, as there are so many possible peptides that every position occupied by an amino-acid may presumably be overlapped by unknown peptides. These may give typical ninhydrin reactions but can be decomposed on suitable hydrolysis. For this reason no

certain identification is possible by simple matching of position of substances which are unstable to hydrolysis. The writer's confusion of glutamine with a peptide containing serine and glycine serves as a personal reminder of this fact.

When the unknown spot in the hydrolyzed solution has been matched with that of a known amino-acid, the next point to be considered concerns the fact or likelihood of another simple substance moving to the same place. This is a far more serious possibility with amino-acids containing more than four carbon atoms (i.e. valine and upwards), since there is a greatly increased chance of isomeric or of totally unrelated substances coinciding. Hence with such substances the identity should be confirmed by isolation by the cutting-out technique (Dent, 1947*a*; Consden *et al.* 1947) followed by matching in other solvents. So far, butanol is the only solvent that need be regularly used for this purpose, but the use of any number could be envisaged in case of ambiguity, since it is little trouble to isolate enough for 10–20 trials.

Finally, other tests, more specific than the ninhydrin reaction, can be applied directly to the spot on the paper, as described for histidine, tyrosine and citrulline. Stability to H_2O_2 would also exclude the presence of dicovalent sulphur. Basic amino-acids are greatly slowed in phenol by acetic or hydrochloric acid vapours. There is much scope for the development of further tests for individual substances.

Bearing all this in mind it must still be admitted that there remains a possibility of confusion between a well-known substance and another whose presence has not been suspected and which may happen to coincide on the chromatogram. The risk is believed small and will diminish as our knowledge of natural substances increases.

It will be noted that two-dimensional chromatograms are exclusively recommended when a mixture of quite unknown composition is to be analyzed. One-dimensionals are only safe when the mixture contains a limited number of amino-acids all of known composition and whose behaviour on the paper has already been investigated, as in the analysis of many protein hydrolysates. For several reasons, one-dimensional chromatography is not safe in the investigation of the non-protein nitrogen fractions of tissues or in the analysis of blood and urine, in all of which new amino-acids have been found with embarrassing frequency. First, there is the phenomenon of 'double overlapping', illustrated in Fig. 3. Many instances of this have been encountered in practice. Secondly, there is the impossibility when the mixture contains very many amino-acids of obtaining good separation of the amino-acids from each other on one-dimensionals. This may be readily surmised from the published photograph of the two-dimensional paper chromatogram obtained by Dent *et al.* (1947) from an extract of potato tuber. This extract contained at least 24 ninhydrin-reacting substances, probably all amino-acids. Finally it is often desired to run very large amounts of the mixture on the chromatogram in order to show up constituents present only in relatively small quantities. This can be done on two-dimensionals well beyond the point where in one-dimensionals the same quantity would show as a continuous streak of colour which would be quite impossible to interpret.

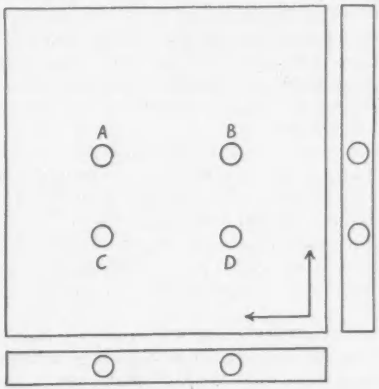


Fig. 3. Diagram of paper chromatograms drawn to illustrate the phenomenon of 'double overlapping'. The four circles (*A*, *B*, *C* and *D*) represent the positions occupied by four hypothetical amino-acids after running as usual on the paper square. One-dimensional chromatograms in phenol and collidine, run with the solvents in the same directions as on the square, are shown below and to the side respectively. In such a case the square would readily resolve a mixture containing some or all of the substances *A*, *B*, *C* or *D*. The two one-dimensionals if run instead of the square would show no qualitative differences between mixtures containing *A* and *D*; *B* and *C*; *A*, *B* and *D*; *A*, *C* and *D*; *B*, *A* and *C*; *B*, *D* and *C*; *A*, *B*, *C* and *D*, since all would appear to contain only two substances. Similar difficulties occur with mixtures only likely to contain three substances, provided that they move to positions similar to three of those in the figure, and in a mixture containing twenty or more amino-acids many instances of double overlapping may occur at the same time on different parts of the chromatogram.

The comparison of an unknown substance with a pure compound which is believed to be identical can be made extraordinarily accurate when the latter reference compound can be obtained in radioactive form. The latter is added to the mixture in trace amounts before they are run together on the chromatogram. The final position of the unknown can then be developed as usual with ninhydrin, and the position of the added pure substance determined by means of a radio-autograph, i.e. by holding the paper against a photographic plate for a sufficient period of

time. The two compounds are thus developed independently of one another. If they are one and the same they will coincide exactly, not only in their average positions but also in the size and shape of the spot. The slight irregularities which always occur in the shape must be exactly reproducible. The final identification has the finality of a 'finger-print' method. This method has been used already by Fink *et al.* (1947) in the case of diiodotyrosine containing ^{131}I.

In practice, with substances of natural origin and the solvent combination described here, the only real confusion in the identification of simple amino-acids in the absence of peptides occurs in the cases of leucine and isoleucine and of citrulline and β-alanine. The methods for distinguishing these are given in the experimental section.

It is conceded that actual isolation in quantity followed by the classical methods of chemical identification should also be undertaken when possible, and is advisable as an independent method of confirmation when claims of the discovery of new amino-acids are made. However, it is held strongly that paper chromatography has a place of its own in amino-acid problems, at least as important as that of the older methods. The latter are themselves often subject to serious criticism when applied to substances separated from mixtures containing many closely related compounds. Certainly as far as concerns the investigation of mixtures, the chromatographic method is far more specific than any chemical or microbiological method in use at the present time.

Finally, it is urged that a new name be found for a technique which is bound to have wide application as a general method of chemistry and which can only be adequately described now as 'partition chromatography using filter paper'. The name 'papyrography' is submitted for consideration.

SUMMARY

1. The two-dimensional filter-paper method of partition chromatography has been applied to some sixty amino-acids and other ninhydrin-reacting substances, using the pair of solvents, phenol and 'collidine'.

2. A map is shown giving the positions taken up by these substances on the paper square.

3. With additional tests the method can be applied to the identification of all the ninhydrin-reacting substances likely to be present in biological fluids.

4. The ninhydrin colour reaction as used here is positive for many aliphatic amines and also for β-, γ-, δ-, and ϵ-amino-acids.

5. Peculiarities in the behaviour of glutamic acid, and to a lesser extent of aspartic acid, suggest that there are unknown facts in the chemistry of these substances.

6. Deaminized casein when hydrolyzed contains only a trace of lysine and an ample quantity of α-amino-ϵ-hydroxycaproic acid.

7. Apart from the common amino-acids the following substances have been found in biological fluids by this method of analysis: β-alanine, α-amino-*n*-butyric acid, γ-aminobutyric acid, ethanolamine, ethanolamine-phosphoric acid, histamine, methionine sulphoxide, methylhistidine. In addition, mention is made of four common but unidentified substances that have been encountered. One ('fast-aminobutyric acid') appears to be a monoaminomonocarboxylic acid, another ('fast-arginine') a new basic amino-acid.

Grateful thanks are due to the many people in Britain and America, too numerous to mention by name, who have donated some of the very valuable specimens used here, and without whose co-operation this work would have been impossible.

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Some Factors affecting the Extraction of Nitrogenous Materials from Leaves of Various Species

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On the basis of experiments with tobacco, Crook (1946) has worked out a technique for the extraction of nitrogenous materials from leaves. Its usefulness has now been tested on leaves from other plants, although no attempt has been made to apply the technique to a comprehensive selection of plants. Those used had been brought to the laboratory for other studies or were conveniently available from local sources. At the same time it has seemed desirable to review and analyze the data which have accumulated over the last 5 years on the effect of certain factors such as the water and nitrogen contents of the leaves on the extraction of nitrogenous materials from them.

The literature relating to nitrogenous materials of leaves and methods of extracting them has been reviewed by Vickery (1945) and in the previous paper (Crook, 1946). More recently Wildman & Bonner (1947) have described some of the properties of proteins extracted from green leaves by a method differing somewhat from that used here.

EXPERIMENTAL

Leaves. Many species were available from the glasshouses. Fig and all cucurbits except marrow, cucumber and bryony were from the University Botanic Garden, Cambridge. The remainder were obtained from plants growing wild or in gardens in the vicinity of Rothamsted. The source is indicated for each species in the tables. No attempt was thus made to control the manurial treatment, age, etc., of the plants, but for certain experiments, advantage was taken of leaf material from fertilizer experiments with tobacco, potato and wheat in which combinations of N, P and K had been applied.

Extraction. The general procedure was that outlined by Crook (1946). The leaves were minced in a domestic meat mincer, squeezed through madapollam, re-minced and washed. In certain instances, e.g. strawberry and artichoke, very little or no sap was obtained after mincing. The mince was then washed three or four times with two or three times its weight of water. The washed fibres were then ground in the triple roller mill previously described (Bawden & Pirie, 1944) and again extracted with water. Neutralization of fibre to pH 8·0 with 0·2 N-NaOH was, in most instances, carried out before milling, but it was sometimes convenient to neutralize after milling. N and dry matter extracted during neutralization were included with the mill extract. The leaf fibre of most cucurbits and certain other species became alkaline on washing (Holden, 1948) and did not require neutralization. Results for these have been listed separately.

Analyses. Dry-matter determinations were made on portions of extracts by drying overnight at 95–100°. N was determined by a micro-Kjeldahl procedure (Crook, 1946).

RESULTS

Extraction of nitrogen and dry matter from various species. In Table 1 are summarized the data on the extraction of nitrogen and dry matter from 28 species distributed among 12 families. The disproportionate number of cucurbits (eight species) was included because these plants had been collected to study the 'alkaline drift' phenomenon (Holden, 1948). Species showing this effect are collected together in § (*b*) of the table, § (*a*) being for leaves whose fibre requires addition of sodium hydroxide to bring the pH to 8·0.

As was to be expected, the efficiency of the extraction process varies considerably for the different species. Extraction of nitrogen ranges from 93½ % with tobacco to 41½ % with nettle, and of dry matter from 78 % (bryony) to 38 % (horse tail). The differences are much more pronounced in mill than in mincer extracts. No consistent differences are noticeable between the two groups of plants. Indeed, § (*b*) of the table contains the two plants showing greatest and least extraction of nitrogen after milling (bryony and calabash). In general, more nitrogen and dry matter are found in mincer than in mill extracts. Strawberry, laurel, comfrey and bryony are the only species for which this is not true of nitrogen. The last two of these are also exceptional in having more dry matter in their mill extracts than is obtained after mincing.

The ratio of nitrogen in the soluble fraction to that in the material sedimentable from any extract at 3000 r.p.m. may vary widely, for the latter may form a small proportion of the extract as in fig (8½%) or may constitute the major fraction (57 %) as in chrysanthemum. However, as there were no obvious regularities in the ratio and as figures for sedimentable material would have complicated the tables unduly, only total extractions have been shown.

The amount of sodium hydroxide required to neutralize the fibre of leaves quoted in § (*a*) of Table 1 varies considerably, and on this basis the plants can

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Table 1. *Extraction of nitrogen and dry matter from leaves of various species*

Family	Latin name	Common name	Source*	Dry matter (% of wet wt.)	N (% of dry matter)	N (% of total)			Dry matter (% of total)		
						In extracts		In residue	In extracts		In residue
						Mince	Mill		Mince	Mill	
		(*a*) Plants whose fibres require neutralization									
Compositae	*Senecio vulgaris*	Groundsel	*W*	8·2	4·68	56	36	8	45	26½	28½
	Chrysanthemum hortorum	Chrysanthemum	*W*	18·6	2·06	31	31	38	41	20	39
Cruciferae	*Brassica oleracea*	Cabbage	*W*	9·7	4·47	55	30½	14½	52½	13½	34
Rosaceae	*Fragaria vesca*, var. Mayo	Strawberry	*W*	33·0	2·84	33	38	29	33	31	36
	var. Royal Sovereign	Strawberry	*W*	36·1	1·57	39½	—	(60½)	43½	—	(56½)
	Prunus laurocerasus	Laurel	*W*	28·8	1·75	21	31	48	30	16½	53½
Cucurbitaceae	*Ecballium elaterium*	Squirting cucumber	*C*	11·8	4·52	47½	42½	10	45½	25	29½
Leguminosae	*Phaseolus vulgaris*	Bean	*G*	14·1	2·80	54	19	27	49½	10	40½
Equisetaceae	*Equisetum* sp.	Horsetail	*W*	17·1	3·23	37	22	41	25	13	62
Gramineae	*Triticum vulgare*	Wheat	*W*	11·6	4·46	67	25	8	47½	17	35½
	Dactylis glomerata	Cocksfoot	*W*	27·0	2·49	35	34	31	28	15	57
Solanaceae	*Physalis alkekengi*	Winter cherry	*G*	23·7	4·03	47	17	36	30	17	53
	Nicotiana glutinosa	—	*G*	9·8	2·76	58	24	18	61½	12½	26
	Nicotiana tabacum	Tobacco	*G*	9·5	4·35	51	42½	6½	50	24	26
	Datura stramonium	Thorn apple	*G*	13·4	4·66	55	38	7	52	27½	20½
	Lycopersicum esculentum	Tomato	*W*	13·5	3·26	61	22	17	58	15½	26½
Buxaceae	*Buxus sempervirens*	Box	*W*	52·1	3·29	45	38	17	31	21	48
Moraceae	*Ficus carica*	Fig	*C*	20·2	4·52	50	37½	12½	38	19	43
		(*b*) Self-neutralizers									
Compositae	*Helianthus annuus*	Sunflower	*W*	17·6	3·24	46	10	44	36	19	45
	H. tuberosus	Artichoke	*W*	18·9	2·49	35	9½	55½	45	7½	47½
Boraginaceae	*Symphytum officinale*	Comfrey	*W*	13·2	4·14	24	28½	47½	21½	42	36½
Urticaceae	*Urtica dioica*	Nettle	*W*	21·8	3·75	29	12½	58½	22	8½	69½
Cucurbitaceae	*Cucurbita ovifera*	Marrow	*W*	14·7	4·66	53½	21½	25	38½	13	48½
	C. ovifera	Marrow	*W*	13·2	2·95	56½	12½	31	41	5	54
	Cucumis sativus	Cucumber	*W*	9·8	4·38	52½	20½	27	42½	17½	43
	Bryonia dioica	Bryony	*W*	11·1	4·60	36	52½	11½	34	—	—
	B. dioica	Bryony	*W*	11·5	6·21	40½	48½	11	37	41	22
	Lagenaria leucantha	Calabash	*C*	11·4	4·21	51½	3½	45	54	3½	42½
	Thladiantha sp.	—	*C*	13·9	3·17	72	5	23	45	5	50
	Cyclanthera explodens	—	*C*	12·0	4·85	58½	28½	13	45½	16	38½

* Source: *G* = glasshouse, *C* = Cambridge Botanic Garden, *W* = wild and garden.

be divided into two groups. In one are those species, among which is included tobacco, whose fibre takes up moderate amounts of alkali—30–60 ml. 0·2 N-NaOH/100 g. In the other are those, such as strawberry and chrysanthemum, which take up very large amounts—200 ml./100 g. and more. The uptake of alkali in the former, which is due chiefly to the demethylation of pectin by pectase (Holden, 1945), proceeds rapidly at first and then more slowly, and is substantially complete in 24 hr. In the latter group, however, the uptake continues at a steady rate for as long as 6 days. Further, there is a simultaneous darkening in colour which does not occur with tobacco-like species and which suggests that oxidation of phenolic substances is concerned.

Relation between nitrogen and water contents of leaves. A review of the early data extending over the period 25 March 1942 to 26 September 1944 on the nitrogen and dry-matter contents of 20 batches of tobacco leaves showed that there was a linear relation between their nitrogen and water contents. In Table 2A are shown the constants for the best straight line through the experimental points calculated by the method of least squares. Application of Fisher's 't test' to this data gave a value of $t = 8{\cdot}86$. The 't test' measures the probability of a random set of data conforming to a hypothesis *by chance*, i.e. the probability that the data here presented should fall *by chance* on the calculated line with the present degree of exactitude. The value of t here obtained indicates that there is less than 1 chance in 10^6 that this is a random result. Subsequent data for tobacco from three manurial experiments (February and March 1946) treated with all combinations of N, P and K did not give results of such high significance, t being reduced from 8·86 to 2·62. No explanation for this difference can be given, but it should be noted that, throughout the early experiments, manurial treatment had been 'normal', whereas the later batches included many plants deficient in N or P. This did not appear to affect the other correlations noted below. Twenty batches of potato leaves from manurial experiments showed no relation between the nitrogen and water contents. However, when all species were considered together, a significant relation was again obtained, t being such that the probability of a chance result of this type was less than 0·001.

Effect of water and nitrogen content of the leaves on the extraction. Early experiments with tobacco suggested that more nitrogen was extracted from the wetter leaves. This was subsequently confirmed and was also found to hold for all species (Table 2B).

The factor of most significance for the extraction of nitrogenous materials was the initial nitrogen content of the leaves. No difference was detectable between the earlier and more recent data for tobacco, and the high positive correlation between nitrogen content and nitrogen extraction also held for all species together. The correlation was as good for extraction by mincing alone as for those by the whole procedure.

It has been found most convenient to express both extracted nitrogen and total nitrogen as a percentage of the total dry matter of the leaf. On this basis, Fig. 1 has been plotted from the combined data for the tobacco experiments referred to above and is included as an indication of the exactness of these relations. The lines shown are those calculated by the method of least squares.

Table 2. *Constants of the regression lines of the form $y = a + bx$ expressing the relations between extraction of nitrogenous materials from leaves and their nitrogen and water contents*

Material or fraction	No. of exp.	a	b Value	b Standard error	t*
A. y = total N (as % dry matter) x = g. water/g. dry matter					
Early data for tobacco	20	1·39	0·16	±0·02	8·86
Later data for tobacco	24	−1·07	0·57	±0·22	2·62
Data for all species	31	2·33	0·24	±0·06	3·92
B. y = extractable N (as % dry matter) x = g. water/g. dry matter					
Tobacco—total extraction	24	−1·78	0·60	±0·20	2·94
All species—total extraction	30	1·20	0·28	±0·07	3·93
C. y = extractable N (as % dry matter) x = total N (as % dry matter)					
Tobacco: Mincer extraction	24	−0·28	0·69	±0·04	16·1
Total extraction	35	−0·34	0·95	±0·04	21·7
All species: Mincer extraction	22	−0·45	0·62	±0·08	7·6
Total extraction	31	−1·14	1·06	±0·10	10·1

* Calculated by Fisher's 't test'. From this value and the number of experiments can be obtained the probability of the experimental points falling on the calculated straight line by chance. For the number of experiments shown here, values of t greater than 2·5 indicate odds of more than 50 to 1 against it being a chance result, and for t greater than 3·5 the odds are more than 1000 to 1 against.

In Table 2C are collected the expressions relating extractable to total nitrogen, both for the tobacco data included in Fig. 1 and for all species listed in Table 1 together. As can be seen, all are highly significant, the values of t being such that the probability of obtaining any one by chance is much less than 1 in 10^6.

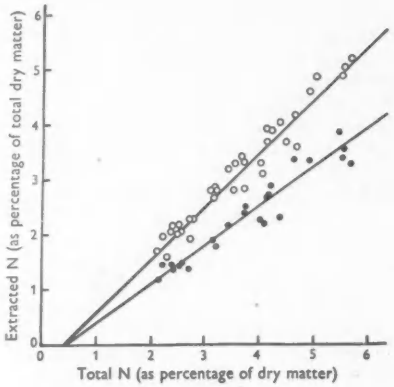

Fig. 1. The relationship between total and extractable nitrogen in tobacco leaves. ●——● extraction after mincing; ○——○ extraction after mincing and milling.

DISCUSSION

There are interesting differences in the ease of handling of various species of plants and in the character of the products. Few regularities of behaviour are observed among related families or even among the members of a single family. In general the wetter and less fibrous leaves are more easily worked although there are important exceptions, e.g. fig and bryony. We have also encountered certain unexplained differences within a single species, e.g. *Ecballium* is given among those plants requiring neutralization. Later samples of this species, however, showed an alkaline drift like other members of the same family.

As a rule, milling is not difficult with any species, provided the fibre is brought to a suitable moisture content. The leaves of some trees are exceptions to this, e.g. those of beech and horse chestnut are not only difficult to mince and give little or no sap on squeezing, but the fibre is so tough and stringy that it will not pass through the triple roller mill. Other plants, such as *Tilia*, contain substances that render their manipulation difficult. This species gives no sap, and, after the addition of water, becomes so slimy and viscous that it is impossible to squeeze extract through the cloths used.

The mincer extracts of several species, e.g. beech, dock and strawberry (var. Royal Sovereign), appear to be free from protein, i.e. give no precipitate with trichloroacetic acid. Bawden & Kleczkowski (1945) have observed the same phenomenon with this variety of strawberry and ascribed it to precipitation of protein by the large amounts of tannins in the extract. The difference in behaviour of even closely related plants is illustrated by a comparison of the two strawberry varieties. Although the amount of soluble nitrogen in mincer extracts of each is very similar, 53% is precipitable from Mayo extracts with trichloroacetic acid.

From the slope and position of the regression lines of Fig. 1 and Table 2C, several conclusions can be drawn. It can be calculated that the average total extraction of nitrogen was 86% from tobacco and 75% from all species. The figure for tobacco is lower than that reported by Crook (1946), but this was based on a procedure involving milling the fibre two or three times. In addition, the average nitrogen content of the leaves on that occasion was rather higher. Both these factors would tend to increase the extraction.

These regressions also make it possible to predict approximately, on the basis of the nitrogen content of the original leaf, the extraction likely to be reached, not only for tobacco, but for any species listed in Table 1. Thus, it can be calculated that, on the average, 78% of the nitrogen can be extracted from a tobacco leaf, 2% of whose dry matter is nitrogen, whereas the extraction rises to 90% for leaves with 6% nitrogen. The differences are greater when the data for all species are considered, the increase being from 48% extraction with leaves containing 2% nitrogen to 86% with those containing 6% nitrogen.

The lines are similar in slope and position both for the extensive data for a single species (tobacco) and for a random sample of plants of various species taken without regard to age, season, manurial treatment or botanical relationship. This perhaps suggests that the observed values are universally applicable and that, if separate regression lines were available for each species, they would all be very similar to that of tobacco.

When extrapolated back, the lines of Table 2C do not pass through the origin, but through points on the total nitrogen axis. This can be regarded as implying that, provided the equation still holds, leaves of this small nitrogen content would show zero extraction, or, what amounts to the same thing, it can be regarded as an illustration of the experimental fact that the last traces of nitrogen are extremely difficult to remove from fibre. As the slopes of the lines for total extraction do not differ significantly from unity, all the nitrogen over and above this limiting quantity is being extracted whatever the initial nitrogen content of the leaf. If the residue could be regarded as 'structural' and unaltered by

grinding and extraction, the picture would be simple —all nitrogen other than this would be extracted. However, the work of Bawden & Pirie (1944) and Crook (1946) suggests that the unextractable residue represents a cross-section of the nitrogenous material present in the cells fixed there by the extraction process itself. The plant cell contains a complex mixture of proteins of varying solubility and in various states of dispersion. These might be expected to be extracted at different rates and to different degrees of completeness. It is surprising therefore that extraction from all species of plants should proceed in such a manner that the residue is representative of all proteins and yet at the same time, the degree of extraction is so accurately expressed by a linear regression.

SUMMARY

1. The extraction of nitrogenous materials from the leaves of various plant species has been investigated by a procedure previously worked out.

2. The extraction varied from 41½ to 93½ % of the total leaf nitrogen, the average for all species being 75%.

3. The extraction was mainly influenced by the nitrogen content of the leaves, increasing with increasing nitrogen content.

4. The extraction was also influenced by the dry-matter content, as the wetter leaves have a higher nitrogen content.

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A Manometric Method for the Estimation of Milligram Quantities of Uronic Acids

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The most widely applicable method for the estimation of uronic acids is that based on the demonstration by Mann (1894) and Mann & Tollens (1896), that uronic acids are decomposed by heating with 12% (w/w) HCl giving a 25% yield of CO_2 (calculated to the anhydride). The method was further explored by Lefèvre (1907) and Lefèvre & Tollens (1907).

In the latter method, the sample was boiled with 12% HCl under reflux for 3·5 hr., and a stream of CO_2-free air passed through the apparatus. After washing and drying, the CO_2 carried from the flask was absorbed in a tube containing KOH. The CO_2 evolved was estimated by the increase in weight of the absorption tube. In subsequent years, many modifications of the original method were introduced, mainly concerned with alterations in the time of decomposition sometimes extended to 5 hr. and even to 8–10 hr. (Ehrlich & Schubert, 1929), and in the methods employed for washing, trapping and estimation of the CO_2. Barium hydroxide became a favoured absorption agent, and titrimetric methods were substituted for gravimetric (cf., for example, Dickson, Otterson & Link, 1930). More recently, attention has been directed to the use of stronger acid to shorten the time needed for decomposition (Colin & Lemoyne, 1938*b*, 18% HCl; McCready, Swenson & Maclay, 1946, 19% HCl), and to measurement of rates of CO_2 evolution in order to estimate traces of uronic acid derivatives in the presence of polysaccharides (Norman, 1939; Whistler, Martin & Harris, 1940).

The most radical departure in the determination of uronic acids by means of the CO_2 evolved on decomposition is that of Voss & Pfirschke (1937), who used an approximately 20 M-solution of $ZnCl_2$ at its b.p. of 146–147° instead of HCl. All these methods have in common the disadvantage of a complex apparatus and a long decomposition time, usually 5–8 hr.

The method to be described is based on the decomposition of not more than 50 mg. of the sample with 0·25 ml. of 12% HCl in a sealed tube at 111°. The CO_2 evolved is then measured by a modification of the method of Van Slyke & Folch (1940) for manometric carbon determination. The present method has the advantage that samples do not need attention during decomposition, and that, as a consequence, determinations may be made at the rate of 4/hr. for long periods.

METHOD

Preparation of tubes. Soda-glass tubing, 7 mm. external diameter, 5 mm. internal diameter, is cut into lengths of 8–10 cm. and cleaned by immersion in cleaning mixture for 3 hr. The tubes are washed, rinsed in distilled water, dried, and a bulb of diameter *c.* 15 mm. blown on the end of each. If carbonate-CO_2 measurements are to be made, the side wall o fthe tube above the bulb is softened in the flame and a pocket of capacity about 0·5 ml. is blown. The tubes are numbered with a commercial black glass-marking ink, dried in the oven, and stored in a desiccator. Bulbs prepared in this way have a volume of about 1 ml., and a total volume of about 2 ml. after sealing; 60–80 bulbs can be blown/hr.

Weighing of sample. The sample should not exceed 50 mg. in weight. It must be deposited in the bulb with no contamination of the walls of the tube, otherwise charring will occur on sealing the tube, and extraneous CO_2 will be formed. A convenient spatula for the introduction of solids into the bulb may be made by sealing a V-section piece of 'sealing-in metal' (obtained from an old radio valve) into a piece of glass tubing that is a sliding fit in the sample tube. If the tubing from which the spatula handle is made fits well, it is almost impossible to deposit material on the walls of the bulb tube if the metal portion is not overloaded. The spatula is introduced holding the tube and spatula horizontal and, when the metal scoop is inside the bulb, it is rotated through 180°, thus depositing the sample, turned to its original position and withdrawn. Liquids are introduced by a capillary pipette. Samples for analysis must be dry. Up to 1 ml. of solutions may be dried off in a weighed bulb and then reweighed.

Addition of acid. HCl (12% w/w) is used. A pipette is made from the tubing used for the spatula handle. The drawn-out tip of the pipette is bent at an angle of 45° from the axis of the pipette, and ground so that it just clears the wall of the tube when the pipette is inserted to the neck of the bulb. This ensures complete delivery and draining of the pipette into the bulb with no loss of acid in the upper parts of the tube. The pipette is fitted with a rubber teat and may conveniently be graduated to deliver four portions of 0·25 ml. If carbonate determinations are being made, the acid is delivered into the side pocket instead of into the bulb.

Decomposition of sample. After the addition of the acid, the tube is sealed off by drawing out in the flame, taking care that a narrow prolongation of at least 2 cm. is secured. In sealing, 'carbonate' tubes must be held horizontal in order to avoid contact between acid and sample. The sealed tubes are secured vertically in a rack, which is immersed in boiling toluene for 5 hr. The toluene may be boiled in the bottom of a tall beaker, into the upper half of which a condenser made from a loose-fitting tin cannister is inserted. Such an arrangement permits the toluene to be boiled vigorously for long periods without loss. After 5 hr., the rack and tubes are removed from the beaker and cooled in water. Twenty-five samples can be weighed into tubes, the acid added, and the tubes sealed ready for decomposition in 1 hr.

Estimation of liberated CO_2. A sealed tube is inserted as far as the bulb into one end of a piece of pressure tubing *c.* 8 cm. long. The other end is attached to the bent tube leading from the gas chamber of the Van Slyke-Neill manometric apparatus. The pressure tubing is exhausted twice by lowering the mercury and expelling the air in the usual way. The second exhaustion serves to verify that there is no leak in the pressure tubing connexion. Two ml. of 0·5 N-NaOH are introduced into the chamber, the mercury lowered to about half-way down the large bulb, and the tap turned to connect the bulb to the chamber. The sealed tip of the bulb is then broken by bending the pressure tubing. The subsequent course of the analysis is substantially that described by Van Slyke & Folch (1940). Ten excursions of the mercury are sufficient for transfer of the CO_2, the process being assisted by tapping the bulb during exhaustion, causing the liquid to coat the walls of the bulb, and warming the bulb in the hand during the mercury upstroke.

Estimation of carbonate CO_2. Samples that contain carbonate are analyzed in the carbonate tubes described above. Two tubes are used for each analysis. In each the HCl is kept from the sample till after sealing, when it is brought into contact with it by bringing to the vertical. One tube is placed in an incubator at 40° for 5 hr. and the other in the toluene bath. The percentage carbonate CO_2 obtained by the analysis of the first tube is subtracted from that found for the second to obtain the uronic-CO_2 value.

RESULTS

Seven samples of galacturonic acid varying in weight from 4 to 12 mg. gave a CO_2 value of 22·33%, S.D. 1·00% (theoretical yield, 22·79%). Since the samples were weighed on a balance reading only to 0·1 mg., these figures show that results are reproducible. Some fifty compounds have been examined for possible interference. The results obtained are listed in Table 1.

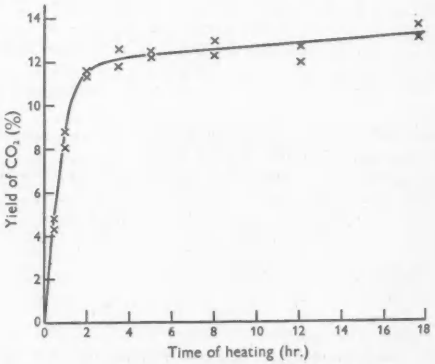

Fig. 1. Variation of CO_2 yield with time of heating; citrus pectin.

Time of decomposition. From Fig. 1, which shows the course of CO_2 evolution in the decomposition of a sample of citrus pectin, it is seen that a period of 5 hr. appears ample for decomposition, and that the continued CO_2 evolution after 5 hr., due possibly to the decomposition of sugar impurities in the pectin and to the further decomposition of the uronic material, is steady, and in very accurate work can be allowed for (Norman, 1939; Whistler *et al.* 1940).

Table 1. *The CO_2 yield of substances tested for interference*

Substance tested	CO_2 yield (mg. CO_2/100 mg. dry substance)	
	Present paper	Other values
Yields >2%:		
Allantoin	cf. Fig. 2	—
Alloxan	8·9, 9·8	—
p-Aminobenzoic acid	cf. Fig. 2	—
Ascorbic acid	17·1, 18·7	—
Hypoxanthine	10·3	—
Pyruvic acid	4·7	5·4(2)
Urea	27·4, 25·6	—
Yields >1%:		
Adenylic acid	1·8, 1·9	—
Agar	1·2	1·04(1)
Tannic acid	1·1	—
Tomato bushy-stunt virus*	1·1	—
Yields <1%:		
Acetic acid	—	Trace (<0·08)(2)
Amylose (potato)	0·65	—
Arabinose	0·6	0·47(8), *c.* 0·7(11)
Asparagine	<0·1	0(6, 7)
*iso*Barbituric acid	0·15	—
Caffeine	<0·1	—
Cellulose	0·3	0·45(7), 0·17(8), 0·16(9)
Cholesterol	<0·1	—
Citric acid	0·2	0(6, 7)
Creatinine	0·3	—
Diethylbarbituric acid	0·2	—
Egg albumin	<0·1	0(6, 7)
Ellagic acid	—	0·12(12)
Erythritol	<0·1	—
Formic acid	—	Trace (<0·08)(2)
Fructose	—	0·55(8), 0·80(7), 0·59(9)
Fucose	0·75	—
Fumaric acid	<0·1	—
Galactose	0·5	—
Gallic acid	—	0(7)
Gelatin	—	*c.* 0·25(11)
Glucono-γ-lactone	—	*c.* 0·25(11)
Glucosamine	<0·1	—
Glucose	0·7	0·18(4), 0·73(6, 7), 0·40(8), 0·19(9), 0·26(10), *c.* 0·25(11)
Glutaric acid	<0·1	—
Gluten (wheat)	0·25	—
Glyceric acid	—	0(2)
Glycogen	0·7	—
Glycollic acid	—	0(2)
Glyoxylic acid	—	0(2)
p-Hydroxybenzoic acid	—	0·53(12)
Inositol	<0·1	—
Inulin	0·85	0·60(8), *c.* 0·25(11)
Lactic acid	—	0·08(2)
Laevulinic acid	0·2	—
Maleic acid	<0·1	—
Malic acid	0·3	*c.* 0·25(11)
Maltose	—	0·40(8)
Mannitol	—	0(8)
Mannose	—	0·55(8)
Melibiose	0·7	—
Melizitose	0·7	—
2:3:6-Trimethylglucose	—	0·20(8)
Mucic acid	0·3	—
Nicotine	<0·1	—
Nicotinic acid	<0·1	—
Nucleic acid (yeast)	0·75, 0·95	—
Oxalic acid	—	0·42(2), 0·8(3), 2(6, 7), *c.* 0·25(11)
Peptone	—	0·4(7), 0·5(6)
Phenolic acids from redwood bark	—	0·58(12)
Phenylethylbarbituric acid	<0·1	—

Table 1 (*cont.*).

Substance tested	CO_2 yield (mg. CO_2/100 mg. dry substance): Present paper	Other values
Yields <1% (*cont.*):		
Rhamnose	—	0·90(8)
Ribose	0·6	—
Salicylic acid	—	0·60(12)
Serine	<0·1	—
Starch:		
Potato	—	0·26(5)
Tobacco (pptd. by ethanol)	—	0·57(5)
Tobacco (pptd. by freezing)	—	0·38(5)
Potato	—	0·30(8)
Rice	—	0·45(8)
Wheat	—	0·40(8)
Horse chestnut	—	0·50(8)
Potato	—	0·20(9)
Potato	—	*c.* 0·25(11)
Commercial soluble	0·65	—
Sucrose	—	0·75(7), 0·78(6), 0·52(8), 0·24(9), *c.* 0·25(11)
Tannin (Mimosa)	0·5	—
Tannin (Quebracho)	0·3	—
Tartaric acid	—	0 (6,7)
Tauroglycollic acid	<0·1	—
Theobromine	<0·1	—
Theophylline	<0·1	—
Thymine	<0·1	—
Tobacco mosaic virus	0·35	—
Trehalose	0·5	—
Uracil	<0·1	—
Uric acid	<0·1	—
Xylose	0·6	0·40(8)

(1) Nanji, Paton & Ling (1925).
(2) Norman & Martin (1930).
(3) Link (1931).
(4) Anderson (1931).
(5) Spoehr & Milner (1935).
(6) Colin & Lemoyne (1938*a*).
(7) Colin & Lemoyne (1938*b*).
(8) Campbell, Hirst & Young (1938).
(9) Norman (1939).
(10) Whistler, Martin & Harris (1940).
(11) McCready, Swenson & Maclay (1946).
(12) Fuller, Bartholomew & Norman (1947).

* Higher value than for tobacco-mosaic virus, probably due to carbohydrate contaminant (Bawden & Pirie, 1938).

Interfering substances. It has been known for some time that substances other than uronic acids or their derivatives evolve small amounts of CO_2 under the conditions used in the determination of uronic acids. One of the earliest statements is that of Dickson *et al.* (1930), who found that the CO_2 yield of various plant acids, sugars, sugar acids, and polysaccharides 'is very small'. Since then, many substances have been tested and the majority have been found to give CO_2 yields negligible for practical purposes (Table 1). A number of substances not previously tested has been found to interfere seriously, however. Some of the previous work has been repeated in order to estimate the magnitude of the effects using the procedure described above, and it will be seen from Table 1 that these effects are of a similar order of magnitude to those found in earlier methods. In addition, other substances have been tested that may be encountered in the analysis of plant material. Some were found to give considerable CO_2 yields under the conditions used. Substances found to give a CO_2 yield over 2% of their weight were *p*-aminobenzoic acid (Weith, 1879), ascorbic acid, allantoin, alloxan, hypoxanthine, pyruvic acid (Norman & Martin, 1930), and urea.

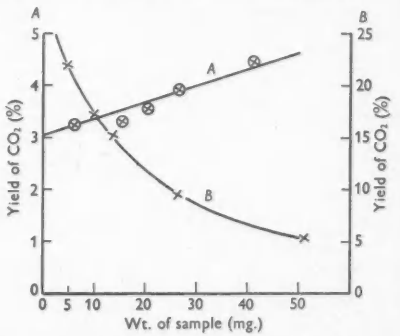

Fig. 2. Variation of CO_2 yield with weight of sample, *A*, *p*-aminobenzoic acid; *B*, allantoin.

When present in material analyzed by the method described, their CO_2 yield may vary with the amount present. Two examples are given in Fig. 2. If it is

suspected that any of these substances are present it is well to include controls with the amount of interfering substance equivalent to that in the sample. Figures for the percentage of CO_2 given by these substances are included in Table 1.

Since the CO_2 yield of *p*-aminobenzoic acid and allantoin varied with the amount used in analysis, it was of interest to determine whether a similar phenomenon was likely to occur with uronic groupings. A series of nine determinations was made on varying weights of the same pectin sample. The average CO_2 yield for five samples (wt. 1·3–9·3 mg.) was 12·4 %, S.D. 1·0 %, while that for four samples (wt. 13·1–18·2 mg.) was 12·0 %, S.D. 0·1 %. Variation in weight of sample used for analysis does not therefore seem to affect the value obtained materially.

DISCUSSION

The accuracy of the method is perhaps not as high as some of the previous methods, but it is well adapted to the routine analysis of small quantities of material. It has been successfully used in this Station for the routine analysis of samples of plant fibre. The method is not well suited to the study of rates of CO_2 evolution carried out by Norman and others (Fuller, Bartholomew & Norman, 1947), in order to distinguish between different polyuronides; but these methods have recently been shown not to yield as clear-cut results as had been hoped (Fuller, 1947). That a number of substances present in plant, animal, or soil material may interfere to a marked extent is of importance, since it has often been assumed that such interference is unlikely to occur. The interfering substances may assume greater quantitative importance in material that has been fractionated; they might, in fact, be concentrated or introduced during the preparation of fractions.

SUMMARY

1. A rapid method for the analysis of small quantities of materials containing uronic acids or uronides is described.

2. The applicability of the method has been gauged by studying the interference by a number of substances, and results compared with those of previous workers.

3. It has been shown that a number of these substances may interfere seriously with the estimation by the present modification of the Lefèvre & Tollens method.

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The Preparation of Sodium Phosphocreatine

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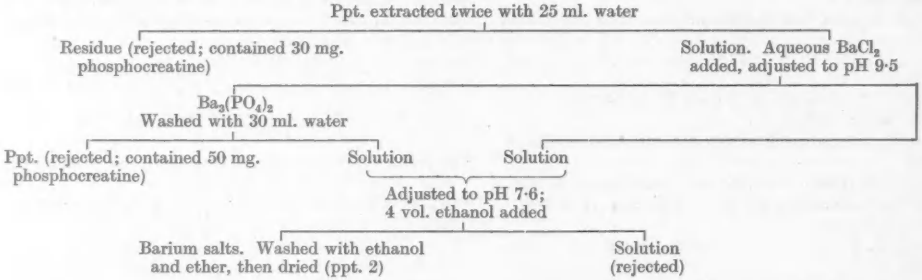
For an *in vitro* study of the metabolic role of high-energy phosphate compounds it is obviously convenient to employ readily soluble sodium salts. This is especially true where the substance has been isolated as the calcium or barium salt and an excess of the anion or oxalate or sulphate is undesirable. In the case of phosphocreatine there is a further complication since, if the barium salt is used, the precipitation of the barium sulphate results in a loss by adsorption of at least 10 % in solutions containing about 5 mg./ml.

The method usually employed for the synthesis of phosphocreatine is that given by Zeile & Fawaz (1938), but, as these authors point out, many times repeated fractional precipitation is necessary in order to obtain the calcium salt free from chloride. We sought therefore an alternative process for the isolation of pure phosphocreatine simpler than that originally devised by Fiske & Subbarow (1929) and followed in principle by Zeile & Fawaz (1938).

Our method depends on the high solubility of sodium phosphocreatine in water, on the insolubility of barium phosphocreatine in 80 % (v/v) ethanol-water, and on the fact that sodium and barium chlorides have a solubility of about 1 % in this mixture.


Ppt. extracted twice with 25 ml. water
Residue (rejected; contained 30 mg. phosphocreatine)
Solution. Aqueous $BaCl_2$ added, adjusted to pH 9·5
$Ba_3(PO_4)_2$
Washed with 30 ml. water
Ppt. (rejected; contained 50 mg. phosphocreatine)
Solution
Solution
Adjusted to pH 7·6; 4 vol. ethanol added
Barium salts. Washed with ethanol and ether, then dried (ppt. 2)
Solution (rejected)

EXPERIMENTAL

Creatine hydrate (20 g.) was triturated with 60 ml. of 10 N-sodium hydroxide, 540 ml. water added and the insoluble residue removed by filtration. Phosphorus oxychloride (60 ml.) and sodium hydroxide (10 N) (400 ml.) were added to the vigorously stirred solution of sodium creatinate over a period of 2 hr. at 0–5°. The product was stirred for 15 min. after the last addition and then filtered. The sodium phosphate was washed by stirring with successive amounts of ice-cold water (200 and 100 ml.); a third extract contained less than 100 mg. of phosphocreatine and was discarded. The combined washings and filtrate were adjusted to pH 7·6 with hydrochloric acid (5 N) and 3 vol. of ethanol (4050 ml.) were added. The precipitated salt contained less than 200 mg. of phosphocreatine and was rejected. The concentration of ethanol in the filtrate was then increased to 80 % (v/v); this resulted in the precipitation of 36 g. of sodium chloride containing an appreciable amount of phosphocreatine. The addition of 1 % (w/v) barium chloride in 80 % (v/v) ethanol to the filtrate precipitated barium phosphate and barium phosphocreatine. The precipitate was separated by centrifugation, washed with ethanol and ether and then dried (ppt. 1). The phosphocreatine in the salt was recovered according to the following scheme:

The combined barium salts (ppts. 1 and 2) were extracted three times with 25 ml. of water adjusted to pH 9·5 by addition of 0·1 N-sodium hydroxide. The residue was suspended in 25 ml. of water and sufficient hydrochloric acid (N) added at 0° to effect solution. Sodium hydroxide (N) was immediately added to bring the pH to 9·5, and the precipitate of barium phosphate was removed by centrifugation and discarded. The supernatant solution was combined with the original washings and barium pre-

* Holding a Research Fellowship from the Wellcome Trustees.

cipitated by the addition of 10% (w/v) sodium sulphate at pH 7·4. Two washings of the barium sulphate were sufficient to remove most of the adsorbed phosphocreatine; the third extract yielded only 22 mg. of phosphocreatine and was therefore rejected. The combined washings and mother liquor now amounted to 220 ml. Ethanol (880 ml.) was added and the mixture allowed to stand at room temperature (21°) for 12 hr. to ensure crystallization. The sodium phosphocreatine separates initially in the form of a suspension of very fine oily droplets, and it is advisable to allow the product to stand undisturbed for a considerable time in order to obtain complete crystallization. When this has occurred the mother liquor is perfectly clear and the phosphocreatine remaining in solution is recovered as the barium salt by the addition of 200 ml. of 1% (w/v) barium chloride in 80% (v/v) ethanol-water.

The first two preparations we carried out gave a product which crystallized in needles and corresponded to a tetrahydrate, but subsequently we have obtained only the hexahydrate which crystallizes in six-sided platelets. The yield of the sodium salt is consistently 3–4 g. and of the barium salt slightly over 1 g. The combined yield of sodium and barium salts is roughly equivalent to the yield of calcium salt reported by Lehninger (1945). We have not achieved the 30% yield claimed by Zeile & Fawaz (1938). The two hydrates of sodium phosphocreatine were completely free from contamination by sodium chloride and inorganic phosphate.

Sodium phosphocreatine tetrahydrate. Found: C, 14·6;* H, 5·4;* N, 12·6; P, 9·7; ash ($Na_4P_2O_7$) 40·4;* creatine 38·5; loss on drying at 70° *in vacuo* 19·2. $C_4H_8O_5N_3PNa_2.4H_2O$ requires C, 14·6; H, 4·9; N, 12·8; P, 10·0; ash, 40·5; creatine 39·9; H_2O, 22·0%.

Sodium phosphocreatine hexahydrate. Found: C, 13·9;* H, 5·4;* N, 11·4; P, 8·4; ash, 36·7;* creatine 35·3; H_2O, 27·8. $C_4H_8O_5N_3PNa_2.6H_2O$ requires C, 13·2; H, 5·5; N, 11·5; P, 8·5; ash, 36·5; creatine 36·0; H_2O, 29·7%.

SUMMARY

A method is described for the preparation of pure sodium phosphocreatine.

* Analyses by Mr F. Hall, Dyson Perrins Laboratory, Oxford.

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The Isolation of Coproporphyrin III from *Corynebacterium diphtheriae* Culture Filtrates

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(*Received* 19 *December* 1947)

It has been recognized that toxic filtrates of *Corynebacterium diphtheriae* cultures contain a red pigment, and in the routine production of diphtheria prophylactic from such filtrates using the alum precipitate method the final preparation is always coloured pink, unless preliminary steps are taken to remove the pigment. Charcoal has frequently been used as a decolorizing agent, but with culture filtrates from the modification of Mueller's (1939) hydrolyzed casein medium, used at the Wright-Fleming Institute for Microbiology for the routine production of diphtheria toxin, the use of charcoal leads to an excessive loss of antigen. Attempts were therefore made to find agents capable of removing the red pigment without serious loss of antigen. A large number of the more commonly used adsorbents were tested without success, but eventually it was found by one of us (L.B.H.) that aluminium benzoate at pH 6·0 and magnesium hydroxide at pH 9·0 were promising. Of these, the latter was found to be more selective, and in general possessed other properties more suited to the preparation of diphtheria prophylactic. The red pigment thus adsorbed on to the magnesium hydroxide proved to be porphyrin in nature, and was readily purified from the magnesium hydroxide precipitate.

The presence of a porphyrin in toxic filtrates of *C. diphtheriae* cultures was first reported by Campbell Smith (1930), and confirmed by Coulter & Stone (1931) who demonstrated the quantitative relation

between porphyrin and toxin production. Most workers (Dhéré, Glücksmann & Rapetti, 1933; Wadsworth, Crowe & Smith, 1935; Pappenheimer & Johnson, 1937) assumed that the porphyrin was a coproporphyrin, and Jakob (1939) claimed, without giving details, to have isolated coproporphyrin III from *C. diphtheriae* as well as from other organisms. However, in further work by Pappenheimer (1947) it was assumed that the diphtherial porphyrin was haematoporphyrin. It therefore appeared necessary to establish its identity on more certain grounds.

The diphtheria porphyrin has proved to be a mixture of at least three porphyrins, but 98–99% consists of coproporphyrin III, the remainder being uroporphyrin I and a hitherto undescribed porphyrin.

EXPERIMENTAL

The lability of the crude toxin makes it preferable, when preparing the porphyrin as a by-product in the large-scale production of diphtheria prophylactic, to perform the magnesium hydroxide precipitation after conversion of the toxin to toxoid.

Preparation of the toxoid. The Park-Williams no. 8 strain of *C. diphtheriae* was grown on the casein-hydrolysate medium described by Mueller (1939), except that the concentration of nitrogen was reduced to 0·13 g./100 ml. (Holt, 1948). After 7 days' growth, the micro-organisms were filtered off and the toxin-containing supernatant liquid treated with 0·4% by volume of 40% formaldehyde solution. After standing at room temperature for 2 days, the reaction was adjusted to pH 7·5 with 10N-NaOH and the solution incubated at 32° for a further 28 days. The resulting crude toxoid solution was further treated as follows.

Preparation of magnesium hydroxide gel. 250 g. (approx. 1 g.mol.) $MgSO_4.7H_2O$ were dissolved in 5 l. of CO_2-free water, and to the resulting solution carbonate-free 0·5N-NaOH (approx. 4 l.) was added with continuous stirring until the pH was 10. After standing 7–10 days the clear supernatant liquid was decanted from the sedimented gel, the volume of which was now about 2·5 l. (i.e. the gel was approx. 0·4M).

Separation of the porphyrin. 0·4M-$Mg(OH)_2$ gel (6·5 l.) was added with stirring to 130 l. of the crude formol toxoid solution, the pH of which had been adjusted to 8·4 by the addition of about 500 ml. 5N-NH_3 solution. Spectrophotometric measurement of the original crude toxoid solution showed a content of 5·8 mg. porphyrin/l. (equivalent to a total of 755 mg.). After standing 1 hr., the $Mg(OH)_2$, now red in colour, was separated from the supernatant, which contained less than 0·1 mg. porphyrin/l., and which was subsequently employed for the preparation of diphtheria prophylactic.

Purification of the porphyrin and preparation of its ester. The red precipitate was suspended in 4 l. of distilled water, and dissolved by the addition of about 500 ml. of 10% (w/v) acetic acid, and the pH adjusted to less than 1 by the addition of 500 ml. 10N-HCl. After stirring for 10 min. the mixture was centrifuged, the purple-red supernatant liquid separated and the residue again extracted by standing overnight with 1 l. of N-HCl. The residual precipitate (now dirty-grey in colour) was centrifuged off, and the supernatant liquid added to that obtained by the first acid extraction. The united portions (6 l.) were then adjusted to pH 3·3, first by the addition of 50% (w/v) NaOH and finally with 30% (w/v) sodium acetate solution. The porphyrin separated as a brown flocculent precipitate, and after standing overnight the mixture was centrifuged. The supernatant liquid contained 0·375 mg./l. of porphyrin. The precipitate was dissolved in 4 l. 0·5N-HCl, and after standing 24 hr. the small brown residue was filtered off and rinsed with a small volume of 0·5N-HCl. The porphyrin was then reprecipitated at pH 3·3–3·4 as before. In all, the porphyrin was precipitated in this way four times at its isoelectric point. Finally, after washing with a little 2% (w/v) acetic acid, the precipitate was dried *in vacuo*. The dried porphyrin was dissolved in 150 ml. methanol previously saturated with dry hydrogen chloride. After standing overnight, the deep-red solution so obtained was poured into 2 l. of ice water in a large separating funnel, and repeatedly extracted with 150 ml. portions of chloroform until the aqueous phase was colourless. The chloroform solution (1200 ml.) was successively washed twice with 1 l. quantities of water, once with 1 l. dilute ammonia, and a further three times with 1 l. quantities of water. After filtration through a chloroform moistened paper, the chloroform was evaporated off leaving the crude ester (578 mg.).

Chromatographic purification of the crude ester. The crude ester was dissolved in 200 ml. benzene, and poured on to a column of $CaCO_3$ (4 × 34 cm.), and the chromatogram was developed with benzene. The main porphyrin fraction was adsorbed very weakly and readily passed into the eluate, leaving a brown zone and a second red porphyrin zone at the top of the column. On washing the column with chloroform in benzene (1 : 10 v/v), this second porphyrin zone split into two zones, one of which remained high in the column while the other moved fairly rapidly into the eluate. The remaining porphyrin zone and the brown zone could not be made to pass down the column even with pure chloroform. The adsorbent was allowed to dry, and on extrusion the third porphyrin zone was eluted with large quantities of chloroform containing 1% (v/v) of methanol. The top brown zone was not further examined.

The main benzene eluate was evaporated to dryness, and crystallized twice from chloroform and methanol. 429 mg. of coproporphyrin III methyl ester (m.p. 155–157° with a remelt at 181–182°) were obtained. This material in chloroform showed absorption bands at 622·1, 572·0, 533·7 and 502·5 mμ., intensity IV > III > II > I (Hartridge reversion spectroscope). A further 50 mg. were obtained by working up the mother liquors. After saponification in conc. HCl and dilution to 0·15N in respect of HCl, the spectral absorption curve over the range 400–750 mμ. was identical with that of coproporphyrin I obtained from the faeces of a patient with congenital porphyria. The Cu complex of the ester was prepared by Fischer's (1937) method, and after five crystallizations gave crystals melting at 218° with absorption maxima at 563 and 526 mμ.

Aronoff (1947) has pointed out that the relative intensities of the absorption bands of diphtherial porphyrin in ether, as given in our preliminary communication (Gray & Holt, 1947), are not typical of a porphyrin. We have re-examined the absorption spectrum of our material in ether solution and, although inspection through a hand spectroscope at first sight seemed to confirm our finding, when the spectral absorption curve was plotted with a spectrophotometer, there was no doubt that the relative intensities were in the order IV > III > II > I. It was obvious that with a hand

spectroscope the narrow width of the absorption band in the red led to an overestimate of its intensity. Others have also been misled by the narrowness of the first absorption band; thus Grinstein, Schwartz & Watson (1945) describe the relative intensities of the absorption bands of coproporphyrin I as IV, III, I and II in decreasing order.

The eluate containing the second porphyrin zone was evaporated to dryness, and after recrystallization from chloroform and methanol gave 7·1 mg. of the typical hair-like crystals of uroporphyrin I octamethyl ester (m.p. 288°). In chloroform, this ester showed absorption bands at 625·7, 576·7, 536·5 and 503·6 mμ., intensity IV>III>II>I (Hartridge reversion spectroscope). The third porphyrin zone has not yet been identified.

DISCUSSION

The isolation of coproporphyrin III ester in relatively enormous quantities in the large-scale production of purified diphtheria toxoid provides an abundant and hitherto unexplored source of coproporphyrin III, which will be of the greatest value in the investigation of the metabolic relationships of this material in the intact animal. The production of small quantities of uroporphyrin I provides yet another example of the so-called dualism of the porphyrins, attention to which was first drawn by Fischer (1937). In the light of present knowledge it is not possible to speculate on the significance of this dualism to the economy of the organism. The implications suggested by Pappenheimer (1947) regarding the relationship of the production of porphyrin to toxin formation will be the subject of a separate paper to be published elsewhere.

SUMMARY

Coproporphyrin III tetramethyl ester in large yield and uroporphyrin I octamethyl ester in very small yield have been prepared from toxic culture filtrates of *Corynebacterium diphtheriae*.

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Crystalline Bacterial Catalase*

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In recent years, the trend of enzyme research has shifted from the study of the reactions catalyzed to a study of the chemical nature of the enzymes themselves. This has led in the last fifteen years to the isolation of some forty enzymes in a crystalline or highly purified state, and in many cases to the identification of their prosthetic groups, resulting in a completely new outlook on enzyme chemistry.

Little progress, however, has been made in the study of bacterial enzymes from this standpoint, and up to the present there has not been recorded the isolation of a single bacterial enzyme in a pure state. This is the more regrettable since many interesting enzymes exist in bacteria which have not been found elsewhere, and some bacterial enzymes at least (e.g. lactic dehydrogenase, cytochromes a_1 and a_2) are known to differ greatly from their counterparts in animal tissues. This relative neglect of bacterial enzymes is in part due to purely technical reasons, namely, the difficulties involved in growing the large quantities of bacteria required, and the problem of liberating endo-enzymes from the bacterial cell. The first of these problems is well on its way to solution, but the second is more difficult. Most of the techniques hitherto used for destroying the cell wall and liberating intracellular enzymes (for example, autolysis, vacuum- or acetone-drying followed by extraction, shaking with glass beads (Curran & Evans,

* A preliminary account of part of this work appeared in *Nature, Lond.*, **160**, 125 (1947).

1942) or grinding with powdered glass (Kalnitsky, Utter & Werkman, 1945), the roller-crushing mill (Booth & Green, 1938), ultrasonic disintegration (Stumpf, Green & Smith, 1946, and others)) either tend to destroy labile enzymes, are difficult to employ on a large scale, or require specialized apparatus.

This paper describes the use of a relatively little-used method of liberating enzymes from bacterial cells, namely, lysis of the bacteria with lysozyme. This substance, which is now easily prepared in crystalline form from egg white, rapidly brings about smooth and complete lysis of susceptible bacteria; no specialized apparatus is required, and the method can be employed on any scale. Penrose & Quastel (1930), Quastel (1937) and Epps & Gale (1942) have used lysozyme to determine the true enzyme content of bacterial cells grown under different conditions, but it has not previously been used as an aid to enzyme purification.

The present paper describes the application of this method to the isolation of catalase from *Micrococcus lysodeikticus*. Catalase was one of the first enzymes to be described in bacteria (Gottstein, 1893) and much work has been done on it since, but no attempts have been made to isolate it in a pure state or determine its chemical nature. Using lysozyme to liberate the enzyme from the bacteria, we have been able to isolate *M. lysodeikticus* catalase as a pure, crystalline protein. As far as we are aware, this is the first bacterial enzyme to have been crystallized.

Bacterial catalase is on the whole very similar to the catalases that have been isolated from mammalian tissues, but there are certain differences. It is a conjugated protein with haematin as the prosthetic group, but unlike liver catalase it contains no verdohaematin. The protein part of the molecule has the same molecular weight as liver catalase protein, but differs in its resistance to organic solvents and low pH. The catalytic activity of the bacterial enzyme is considerably higher than that of blood or liver catalases; this difference also is to be attributed to the protein part of the molecule. Finally, it differs from other catalases in its crystalline form.

METHODS

Bacterial suspensions. The strain of *M. lysodeikticus* used (National Collection of Type Cultures no. 2665) was a descendant of that originally isolated by Fleming (1922). For large-scale production, the bacteria were grown on C.C.Y.-lactate agar (Gladstone & Fildes, 1940) in enamelled trays (photographic developing dishes) measuring 11 × 16 in., with overlapping lids, each containing 500 ml. of agar. Three ml. of inoculum, prepared by suspending the 24 hr. growth from a Roux bottle in 50 ml. of saline, was spread evenly over the surface of each tray with a glass spreader, and the trays incubated at 35°. Growth was almost complete in 24 hr., but the catalase activity of the bacteria continued to increase up to 40 hr., which was therefore adopted as the standard time for incubation. The bacterial growth was then removed from the agar surface with a scraper. The average yield was *c.* 1·5 g. bacteria (dry wt.)/tray.

Estimation of catalase activity. The activity of catalase preparations was measured essentially according to Euler & Josephson (1927). Suitably diluted catalase solution (1 ml., containing *c.* 0·3 μg. of pure enzyme) is added to 49 ml. of 0·015 N-H_2O_2 in 0·015 M-phosphate buffer of pH 6·8, kept at 0° in an ice-water bath. Five ml. are immediately withdrawn, pipetted into 5 ml. of 2 N-H_2SO_4, and titrated with 0·01 N-$KMnO_4$. Further samples are withdrawn and titrated after 3, 6, 9 and 12 min. For each time interval, the first-order velocity constant is calculated as $k = \frac{1}{t} \log_{10} \frac{a}{a-x}$, where a is the initial $KMnO_4$ titre and $a-x$ the titre after t min.

Under these conditions the reaction is approximately first order but, as with catalase of plant and animal origin, significant destruction of the enzyme by the H_2O_2 occurs, causing falling values of k. Following Sumner (1941), we plot k against t and determine k_0, the value of k at zero time, by extrapolation. The amount of catalase taken should be such that k_0 falls between 0·025 and 0·04 $min.^{-1}$; the value of k_0 is then directly proportional to the amount of enzyme taken.

Catalase solutions always have to be highly diluted for testing; the activity of the undiluted solution is calculated by multiplying the value of k found in the test by the overall dilution factor; e.g. if 1 ml. of a 1/10,000 dilution gives a k value of 0·03 in the test, then k for the undiluted solution is $0{\cdot}03 \times 50 \times 10{,}000 = 15{,}000$. The purity of an enzyme preparation is expressed according to Euler & Josephson (1927) as $Kat.\text{-}f. = \frac{k}{\text{g. enzyme in 50 ml.}}$, or under the above test conditions, $Kat.\text{-}f. = \frac{k \text{ in test}}{\text{g. enzyme in test}}$.

To obtain reliable results by this method, it is essential that all glassware should be cleaned with H_2SO_4-$K_2Cr_2O_7$, and that the very dilute enzyme solutions necessary for the test should be used immediately after diluting. Even so, we consider the error to be about 5%. All measurements reported in this paper are the means of duplicates.

Dry weights. Well-dialyzed enzyme solutions, or bacterial suspensions thoroughly washed in distilled water, were dried to constant weight at 105° and weighed on a micro-balance. The colorimetric method of Pressman (1943) was used as a rapid, rough method of determining the protein content of enzyme fractions; all measurements reported, however, are based on direct weighings. Dry weights of bacterial suspensions were as a routine measured turbidimetrically using a Hilger photoelectric absorptiometer previously calibrated with suspensions of known dry weight.

Haemin estimations. The method chiefly used was the pyridine-haemochromogen method of Keilin & Hartree (1936) and Rimington (1942). Results were occasionally checked by the cyanhaematin method of King & Gilchrist (1947); the two methods gave identical results.

Crystalline lysozyme. This was initially prepared from egg white by bentonite adsorption according to Alderton, Ward & Fevold (1945). Once a supply of seed crystals had been prepared, subsequent batches were made by direct crystallization from egg white (Alderton & Fevold, 1946); this method was found very simple and reliable.

Absorption spectra were observed with a Hartridge reversion spectroscope (calibrated with a neon lamp), and a Beckman spectrophotometer.

RESULTS

Action of lysozyme on Micrococcus lysodeikticus *suspensions*

When a dilute suspension (say 1 mg./ml.) of *M. lysodeikticus* in 0·5 % saline is treated with a small amount of lysozyme, the turbidity rapidly disappears leaving an almost water-clear solution in which no intact bacteria are observable microscopically; the phenomenon has been described in detail by Fleming (1922, 1929) and subsequent workers.

An interesting finding is that the catalase activity of *M. lysodeikticus* suspensions invariably increases after lysis with lysozyme, usually by about tenfold. For example, a typical suspension gave the following results (tested at 30°):

	Bacteria in test (mg.)	*k*	*Kat.-f.*
Before lysis	0·424	0·0382	89
After lysis	0·071	0·0606	854

The same effect is observed if the bacteria are disintegrated by shaking with glass beads. Similar results were observed by Penrose & Quastel (1930), and Krampitz & Werkman (1941) found that intact *M. lysodeikticus* suspensions had no action on oxaloacetic acid, whilst lyzed or acetone-dried bacteria rapidly decarboxylated this substance. The simplest explanation is that diffusion of substrate into the cell is a limiting factor.

Fleming (1929) noted that high salt concentrations inhibit lysis by lysozyme. We find also that in the total absence of salts (bacteria washed with distilled water and treated with dialyzed lysozyme solutions) lysis is almost completely inhibited for prolonged periods. The optimal salt concentration is about 0·5 % for NaCl and 0·02–0·05 M for phosphate buffers; under these conditions, 1 mg. of crystalline lysozyme is more than sufficient to bring about complete lysis of 1 g. of *M. lysodeikticus* in 1 hr. at 30°.

Previous workers on lysozyme have only studied its action on quite dilute bacterial suspensions. When large amounts of bacteria in thick (1–8 % dry weight) suspension are lyzed, interesting new phenomena are observed. When acted on by lysozyme, such suspensions of *M. lysodeikticus*, which are originally canary yellow and completely opaque, rapidly become greenish yellow and semi-transparent though not completely water-clear; the residual turbidity is due to the high concentration of 'ghosts'. As these changes take place the initially mobile suspension becomes highly viscous and slimy. The solution produced by lysis of a 1 % bacterial suspension has about the consistency of egg white, while an 8 % solution is a semi-solid gel which cannot be pipetted or poured out of a test-tube; a 4 % solution is the strongest that can conveniently be handled. Besides being highly viscous, such solutions have marked elastic properties. They are readily drawn out into threads, and on pouring from a measuring cylinder emerge as an elongated 'blob' which slowly descends while the cylinder is tilted and runs back into the cylinder if the latter is restored to an upright position. When caused to flow along a 2 mm. horizontal capillary under pressure (in an apparatus similar to that used by Scott Blair, Folley, Malpress & Coppen (1941) for testing the flow-elasticity of bovine cervical mucus), they show marked recoil when the pressure is suddenly released.

The chemical nature of this viscous substance or substances is unknown, nor is it known whether they are initially present inside the bacterial cell and released from it by lysis, or formed by the action of lysozyme; it is hoped to investigate these problems in the future. The phenomenon is not peculiar to *M. lysodeikticus*, similar viscous substances being formed when other sensitive bacteria are treated with lysozyme.

Isolation of crystalline catalase from lysed bacteria

The essentials of the purification method we have adopted are as follows. The solution of lyzed bacteria is treated with 0·5 vol. of ethanol at pH 5·6, which precipitates the viscous substances formed on lysis, and then shaken with chloroform, which denatures considerable quantities of inert proteins. The aqueous-ethanolic solution is then treated with solid ammonium sulphate which, with correct proportions of the three components, causes it to separate into two liquid phases, one containing all the catalase while the other contains most of the contaminating proteins. This 'partition' method is, as far as we know, a novel procedure in protein purification, and may well be applicable to other problems.

After repetition of the above process, two fractionations with ammonium sulphate bring the enzyme to 70–80 % purity, when it can be crystallized either by prolonged dialysis or by careful addition of ammonium sulphate.

The following are the details of a typical preparation:

Stage 1. The bacterial growth from 156 trays (78 l. of medium) was harvested, suspended without washing in *c.* 3 l. of 0·5 % NaCl, and strained through muslin to remove flakes of agar. Turbidimetric estimation showed that the total dry weight of bacteria was 203 g., and 0·5 % NaCl was added to make the final concentration of the suspension 4 % on a dry-weight basis (5085 ml.). Crystalline lysozyme was then added (1 mg./g. bacteria) and the suspension incubated 1 hr. at 30°, when lysis was complete. *Kat.-f.* of lyzed suspension = 910.

Stage 2. The highly viscous lyzed suspension was treated successively with 509 ml. of M-acetate buffer of pH 5·6, and 2800 ml. of ethanol (final ethanol concentration 33·3% v/v). A bulky yellow gelatinous precipitate was centrifuged off, washed with 2500 ml. of M/15 acetate (pH 5·6) containing 33·3% ethanol, and the washings added to the original supernatant fluid. This gave 8180 ml. of a pale yellow mobile liquid; *Kat.-f.* = 4020. (All the above operations were carried out at 0°; this is not absolutely essential, but gives better yields.)

Stage 3. The above liquid was treated with 1820 ml. of chloroform, shaken on a fast mechanical shaker for 15 min. and centrifuged, when two liquid layers formed with a thick layer of denatured protein at the interface. The top layer (7610 ml.) was siphoned off; *Kat.-f.* = 5200.

Stage 4. The top layer from the last stage was treated with $\frac{1}{10}$ vol. of M-sodium acetate and 2400 g. of solid $(NH_4)_2SO_4$ added (30 g. to each 100 ml.). This caused the separation on standing of two liquid layers, the lower containing most of the $(NH_4)_2SO_4$ and the upper most of the ethanol, some $(NH_4)_2SO_4$ and water. The smaller top layer (2760 ml.) was pale brown and contained all the catalase, whose characteristic absorption band at 631 mμ. could now be seen with a hand spectroscope; *Kat.-f.* = 15,650. (It is essential for the success of this step that the proportions of aqueous solution, ethanol and chloroform specified in stages 2 and 3 should be adhered to; otherwise two layers may not separate, or the catalase may be precipitated at the interface. The sodium acetate is added to keep the pH at about 5·6 on addition of the $(NH_4)_2SO_4$.)

Stage 5. The top layer from the above stage was shaken for 15 min. with an equal volume of chloroform, allowed to stand, and the top layer (1890 ml.) removed. (Comparatively little inert protein is removed by this step; the main purpose is to reduce the ethanol concentration.) Solid $(NH_4)_2SO_4$ was now added (23 g. to each 100 ml.) when two layers again separated. The top layer was much the smaller, and dark brown; it was removed in a separating funnel and dialyzed against running tap water overnight; final volume 600 ml., *Kat.-f.* = 20,200.

(Stages 4 and 5 bring about a fourfold purification and decrease the volume to $\frac{1}{12}$; this concentration is important for the subsequent $(NH_4)_2SO_4$ fractionation which is less effective if the protein concentration is too low.)

Stage 6. The dialyzed solution from stage 5 was brought to pH 5·6 by the addition of $\frac{1}{10}$ vol. of M-acetate buffer, and roughly fractionated by adding successive portions of solid $(NH_4)_2SO_4$ and centrifuging off the resulting precipitates. Fraction 6*a* (obtained with 1·96 M-$(NH_4)_2SO_4$) which was greyish white and contained little catalase, was rejected. Fractions 6*b*, *c* and *d*, taken off at 2·27, 2·38 and 2·52 M-$(NH_4)_2SO_4$, were brown and contained most of the catalase, little being left in the final supernatant. These three fractions were combined and dissolved in M/20 acetate pH 5·6 to give a volume of 61 ml., *Kat.-f.* = 40,000.

Stage 7. The combined fractions *b*, *c* and *d* were now carefully fractionated by dropwise addition of a 4 M-$(NH_4)_2SO_4$ solution adjusted to pH 5·6 with NH_4OH. (It was found impossible to standardize these fractionations completely. The exact $(NH_4)_2SO_4$ concentration at which catalase begins to precipitate is markedly affected by the catalase concentration, the temperature and probably other factors. It is necessary to proceed empirically, adding the $(NH_4)_2SO_4$ drop by drop and with good stirring, taking off fractions at intervals. Fortunately, the colour of the catalase makes it easy to determine its distribution in the precipitates.) Three successive precipitates were taken off, after which little catalase remained in the supernatant fluid. The smaller 1st and 3rd precipitates were visibly paler than the 2nd, and obviously contained colourless protein impurities. The 2nd dark brown precipitate contained the bulk of the catalase and was dissolved in M/20-acetate pH 5·6 to give a volume of 63 ml., *Kat.-f.* = 62,900.

Stage 8 (*crystallization*). The solution resulting from the last stage was crystallized in three different ways. (i) A portion (10 ml.) of the solution was dialyzed against repeated changes of distilled water at 0°. After 5 days (longer than this may be necessary) it had mostly crystallized. (ii) Another 10 ml. portion of the solution was cautiously treated with 4 M-$(NH_4)_2SO_4$ until the faintest turbidity appeared. The solution was allowed to stand at room temperature, and by the next day it had almost completely crystallized, leaving a nearly colourless mother liquor. (iii) The rest of the solution (43 ml.) was treated with just enough 4 M-$(NH_4)_2SO_4$ to precipitate all the catalase. The precipitate was centrifuged down and redissolved by adding distilled water drop by drop, very slowly and with good stirring, until all but a small portion had dissolved; this was centrifuged off. The supernatant fluid was then saturated with amorphous catalase; it was allowed to stand at room temperature, and most had crystallized within 24 hr. (iii*a*). A smaller second crop of crystals (iii*b*) was obtained by adding a few drops of $(NH_4)_2SO_4$ to the mother liquor of the 1st crop. The four batches of crystals were separately dissolved in M/15-phosphate buffer pH 6·8, well dialyzed, and tested; *Kat.-f.* = 90,000–98,000, which is the highest value we have obtained. There is thus a considerable increase in purity on crystallization.

The yields and purities at each stage of the isolation procedure are shown in Table 1.

Table 1. *Purification of bacterial catalase*

Stage	Volume (ml.)	Total protein (g.)	Total catalase (g.)		*Kat.-f.*
1. Lyzed bacteria	5,085	203	1·94		910
2. Ethanol supernatant	8,180	40·2	1·69		4,020
3. Chloroform supernatant	7,610	29·5	1·60		5,200
4. First ethanol-$(NH_4)_2SO_4$ partition	2,760	8·9	1·46		15,650
5. Second ethanol-$(NH_4)_2SO_4$ partition	600	5·4	1·14		20,200
6. First $(NH_4)_2SO_4$ fractionation	61	2·1	0·90		40,000
7. Second $(NH_4)_2SO_4$ fractionation	63	0·735	0·485		62,900
8. Crystals: (i)	—	0·050	0·050		93,300
(ii)	—	0·065	0·065	0·376	98,000
(iii*a*)	—	0·206	0·206		99,000
(iii*b*)	—	0·055	0·055		90,000

Properties of the crystalline enzyme

General properties. Solutions of the pure enzyme have a red-brown colour resembling that of methaemoglobin. Strong solutions (1 % or over) are stable for many weeks at 0°, or even at room temperature, if bacterial contamination is avoided, but very dilute solutions such as are used in *Kat.-f.* determinations lose their activity fairly rapidly, and must be tested immediately. In this they resemble liver catalase (Sumner & Dounce, 1937).

Crystalline form. The pure enzyme crystallizes as regular octahedra, which are isotropic when viewed between crossed polaroids; they have the same form, however the enzyme is crystallized (Pl. 3). In this respect bacterial catalase differs from beef-liver catalase, which according to Sumner & Dounce (1937) may crystallize as needles, plates or prisms, according to the conditions of crystallization.

Prosthetic group. Bacterial catalase has a higher activity (*Kat.-f.*) than catalases from other sources (Tables 1 and 3). It is important to discover whether this is to be attributed to differences in the protein

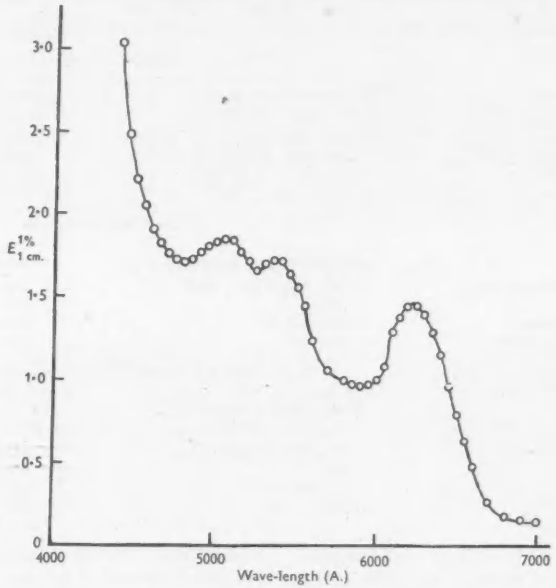


Fig. 1. Absorption spectrum of bacterial catalase. Crystalline enzyme (*Kat.-f.* = 99,000) in M/15 phosphate pH 6·8.

Bacterial catalase is fairly stable at high pH values (e.g. in 0·1N-ammonia) but much less stable to acid pH. Horse liver and erythrocyte catalase are stable down to pH 3·2 (Agner & Theorell, 1946), but bacterial catalase is instantly denatured and precipitated at pH 4·0, and fairly rapidly at pH 4·6. Hence, treatment with acetate buffer pH 3·8, which Bonnichsen (1947) used effectively for purifying mammalian catalases, cannot be employed for purifying the bacterial enzyme. Dioxan, which Sumner & Dounce (1937, 1939) used to purify liver catalase, denatures bacterial catalase very rapidly at room temperature and still rapidly at 0°; we were able to obtain fairly pure preparations by Sumner's method working at 0°, but only in very low yields.

or the prosthetic group, and the nature of the latter was investigated in some detail.

Strong solutions of bacterial catalase have a characteristic absorption spectrum (Fig. 1), showing three bands centred at 506, 545 and 631 mμ. (in M/15 phosphate pH 6·8), which are close to the values reported for other catalases. The exact positions and intensities of the bands are affected both by the pH and the nature of the buffer anion (cf. Agner, 1942). These data are not always recorded in the literature; it is uncertain, therefore, whether the slight spectroscopic differences observed between different catalases are apparent or real.

On treatment with pyridine and sodium hyposulphite ($Na_2S_2O_4$) in 0·1N-NaOH bacterial catalase

gives a typical haemochromogen spectrum, and it forms characteristic compounds with cyanide and azide. Neither azide-catalase nor cyanide-catalase, nor catalase itself, is reduced by hyposulphite. Azide-catalase forms a characteristic pink compound with hydrogen peroxide, which gradually reverts to the original spectrum as the hydrogen peroxide decomposes. This behaviour is exactly similar to that of liver catalase as described by Keilin & Hartree (1937), and the absorption bands of the compounds have similar positions (Table 2).

Table 2. *Absorption bands of catalase and catalase-haematin derivatives*

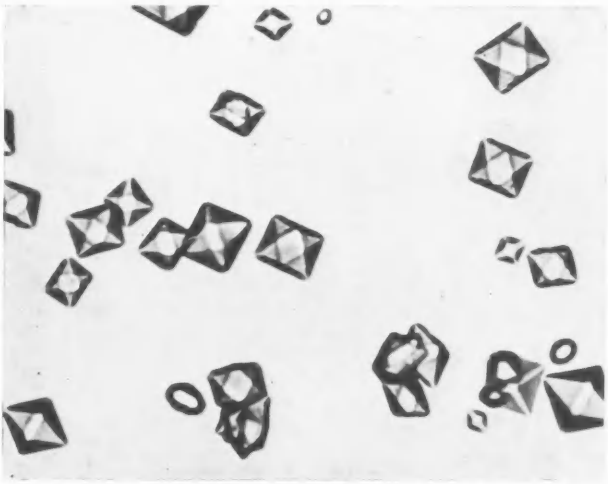
(Positions of the absorption bands, determined with the Hartridge reversion spectroscope, are given in mμ.)

	Catalase derivatives	
	M. lysodeikticus catalase (this paper)	Horse-liver catalase (Keilin & Hartree, 1937)
Catalase	506, 545, 631·5	506·5, 544, 629·5
Azide-catalase	504, 541, 623	506·5, 544, 624·5
Azide-catalase—H_2O_2	548, 588	547, 588
Cyanide-catalase	555·5, 592	556·5, 595·5
	Haematin derivatives	
	Bacterial catalase haematin	Blood haematin
Haematin (in ether-acetic acid)	505, 545, (584), 638	506, 544·5, (585), 637
Cyanhaematin	547	547
Reduced cyanhaematin	537, 578·5	536·5, 578
Pyridine haemochromogen	525, 557	526, 557
	'Resynthesized haemoglobin' derivatives	
	From globin + bacterial catalase haematin	From globin + blood haematin
Methaemoglobin (alkaline)	542, 575·5, 603·5	541·5, 575·5, 603
Haemoglobin	557	560
Oxyhaemoglobin	540, 577·5	540·5, 577
CO-haemoglobin	536·5, 572	537, 572

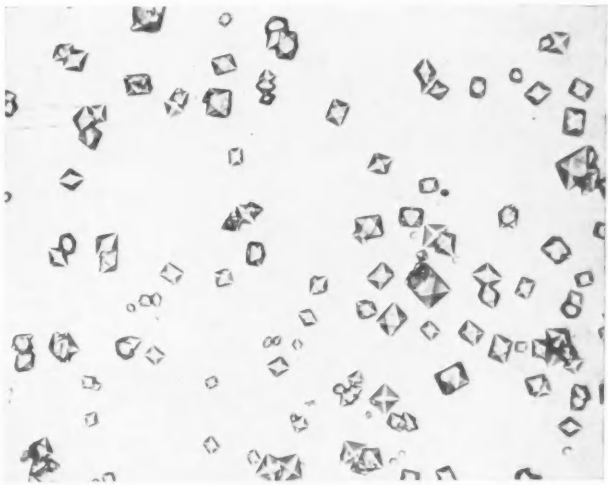
The above facts strongly suggest that the prosthetic group of bacterial catalase is haematin, but further evidence is needed. This was obtained by splitting off the prosthetic group with acetone-hydrochloric acid and coupling the isolated pigment to globin, when it formed methaemoglobin which could be converted to reduced haemoglobin, oxyhaemoglobin and carboxyhaemoglobin, identical with the corresponding derivatives formed from globin and pure haemin. This is proof that the prosthetic group of bacterial catalase actually is haematin; the same experiment also showed that it contains no verdohaematin or similar substance giving rise to biliverdin on treatment with acetone-hydrochloric acid. Details are given below:

Bacterial catalase (15 ml. of 1·5%) was run into 300 ml. of pure acetone containing 5 ml. of conc. HCl. The flocculent protein precipitate was filtered off and washed with acetone-HCl, the washings being added to the original filtrate. The washed protein precipitate was pure white. The acetone-hydrochloric acid filtrate was brown, showing diffuse bands at 502, 547 and 644 mμ., identical with those of haemin in this solvent. It contained no detectable trace of the 'blue pigment' (biliverdin) that is produced when liver catalases are treated in a similar way (Sumner & Dounce, 1939). On evaporating off the acetone *in vacuo*, the haemin precipitated, leaving a colourless supernatant fluid. The haemin was centrifuged off, washed with water, and dissolved in 10 ml. of dilute Na_2CO_3. Part of this alkaline haematin solution was reserved for conversion to the cyanide- and pyridine-haemochromogen derivatives; the remainder was immediately coupled with a *c.* 1·5% solution of globin prepared from human-blood haemoglobin by the method of Anson & Mirsky (1929–30). The globin solution (which was free from denatured globin) was added drop by drop to the solution of catalase haematin until visual and spectroscopic observation showed that all of the latter had coupled with the globin; the solution then showed the absorption bands of methaemoglobin. This was reduced with the minimum quantity of Stokes's reagent (1% $FeSO_4$ in 2% tartaric acid) required to convert it to reduced haemoglobin. On shaking vigorously with air this was converted to oxyhaemoglobin, which was further converted to CO-haemoglobin by saturation with CO. The same series of compounds was formed from the same globin solution by coupling with a freshly prepared solution of recrystallized blood haemin; the absorption bands of both series, and of the other catalase haematin derivatives, are shown in Table 2. The spectra of the two series of haemoglobin derivatives were identical within the errors of reading (Table 2).

For comparison, samples of purified horse-liver catalase and crystalline human-blood catalase were treated with acetone-hydrochloric acid in the same way. The blood catalase behaved exactly like the bacterial catalase, giving a brown acetone solution



(*a*) Crystallized from $(NH_4)_2SO_4$; ×500.



(*b*) Crystallized by dialysis; ×200.

Crystalline catalase from *Micrococcus lysodeikticus*.

containing, as far as could be ascertained, only haematin. The liver catalase, however, gave a bright blue acetone solution, containing both haematin and biliverdin, as Sumner & Dounce (1939) have described. This could be detected with even a few drops of a 1·5% liver catalase solution; bacterial catalase contains none of the substance (probably verdohaematin, see Lemberg & Legge, 1943) that gives biliverdin on treatment with acetone-hydrochloric acid.

Solutions of bacterial catalase and blood catalase are identical in colour to the naked eye, while horse-liver catalase is distinctly greener in colour, and can be seen spectroscopically to have a higher end-absorption in the red. This difference in colour is almost certainly due to the presence of verdohaematin in the liver catalase, and its absence in the other two catalases.

Catalytic activity, haematin content and molecular weight

Table 3 records the *Kat.-f.* and haematin content of crystalline bacterial catalase preparations compared with those of other catalases, and also the ratio *Kat.-f.*/percentage of haematin. Evidently this ratio should be independent of the purity of the catalase preparation provided that impurities present (*a*) contain no haematin, (*b*) have no effect on the activity of the enzyme. Fig. 2 shows *Kat.-f.* plotted against the percentage of haematin for bacterial catalase preparations of varying degrees of purity, the lowest

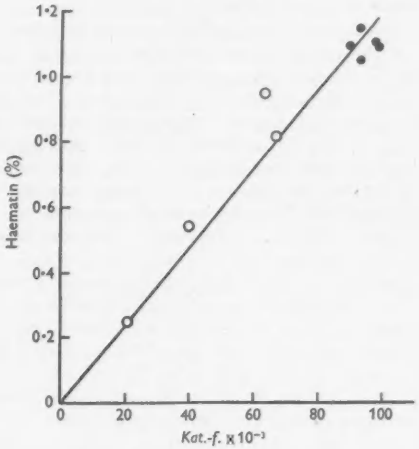


Fig. 2. Relation between haematin content and *Kat.-f.* for bacterial catalase at different stages of purification. Crystalline preparations, ●; amorphous, ○.

only 20% pure. The points are well fitted (within the limits of error) by a straight line passing through the

Table 3. *Catalytic activity and haematin content of bacterial catalase compared with liver and erythrocyte catalases*

Source	*Kat.-f.*	Haematin (%)	Bile pigment (%)	*Kat.-f.*/% of haematin	Sedimentation constant	References
			Liver catalases			
Ox	30,000–35,000	0·46–0·54	0·49–0·55*	57,000–67,000	$11·2 \times 10^{-13}$	Sumner & Dounce (1939); Sumner & Gralén (1938)
Horse	25,000–40,000	0·41–0·68	0·37–0·62*	58,000–61,000	—	Sumner, Dounce & Frampton (1940)
	34,000–48,000	0·67–0·92	+	50,000–53,000	—	Keilin & Hartree (1945*a*)
	50,000	0·89–0·91	+	55,000–56,000	$11·3 \times 10^{-13}$	Bonnichsen (1947), Agner (1938)
			Erythrocyte catalases			
Ox	48,000	?	Nil	?	—	Laskowski & Sumner (1941)
Horse	65,000–70,000	1·042	Nil	62,000–67,000	—	Agner & Theorell (1946)
	65,000	1·08	Nil	60,000	—	Bonnichsen (1947)
Human	50,000	0·83	Nil	60,300	—	Bonnichsen (1947)
	63,000	1·15	Nil	54,700	$11·26 \times 10^{-13}$	Herbert & Pinsent (1948); Cecil & Ogston (1948)
			Bacterial catalase			
M. lysodeikticus	93,300†	1·042	Nil	89,500	—	This paper
	98,000‡	1·103	Nil	89,000	—	This paper
	99,000§	1·096	Nil	90,000	$11·0 \times 10^{-13}$	This paper; Cecil & Ogston (1948)
	90,000‖	1·096	Nil	82,200	—	This paper

* From 'bile pigment Fe' analyses.
† Crystallized by dialysis, stage 8 (i).
‡ Crystallized by $(NH_4)_2SO_4$, stage 8 (ii).
§ Crystallized by $(NH_4)_2SO_4$, stage 8 (iii*a*).
‖ Crystallized by $(NH_4)_2SO_4$, stage 8 (iii*b*).

origin. This shows that our preparations are not contaminated with other iron-porphyrin proteins.

Table 3 shows that the haematin content of our preparations is the same as those recorded by other workers for blood catalases and higher than those reported for liver catalases; the *Kat.-f.*, however, is *c.* 50% higher than the most active catalases of animal tissues, the average value being *Kat.-f.* = 95,000. Similarly, the ratio *Kat.-f.*/percentage of haematin is higher, being *c.* 88,000. To make certain that this is not due to any error in our analytical methods, we prepared crystalline blood catalase by a new method (Herbert & Pinsent, 1948), and determined its activity and haematin content in parallel with determinations on bacterial catalase. Any systematic errors in our *Kat.-f.* and haematin determinations would apply equally to both enzymes. In fact our values for blood catalase (Table 3) are in good agreement with those of other workers. We concluded, therefore, that bacterial catalase actually has a considerably higher activity, whether measured on a dry weight or on a haematin basis, than the catalases of mammalian tissues. Since the prosthetic groups are the same, the difference must be attributed to the protein component of the enzyme.

Assuming our crystalline preparations to be pure, the mean haematin content of 1·09% corresponds to a molecular weight of $58{,}000 \times n$, where n is the number of haematin groups/mol. Assuming $n = 4$, as for catalases of mammalian tissues, this gives a value for the molecular weight of 232,000.

Cecil & Ogston (1948) have examined our crystalline preparations in the ultracentrifuge and found them to consist essentially of a single homogeneous protein with sedimentation constant $11{\cdot}0 \times 10^{-13}$ (Table 3). In the best preparation examined in the ultracentrifuge, the catalase accounted for 85% of the sedimenting material. As the error of this determination is *c.* 5%, we may conclude that our preparations were essentially homogeneous. The haematin content and the agreement between the chemical and ultracentrifugal calculations of the molecular weight lead us to suspect that the small amount of impurity apparently present is probably denatured catalase. If this is so, then the activity of the completely pure enzyme may be some 10% higher than the values given above.

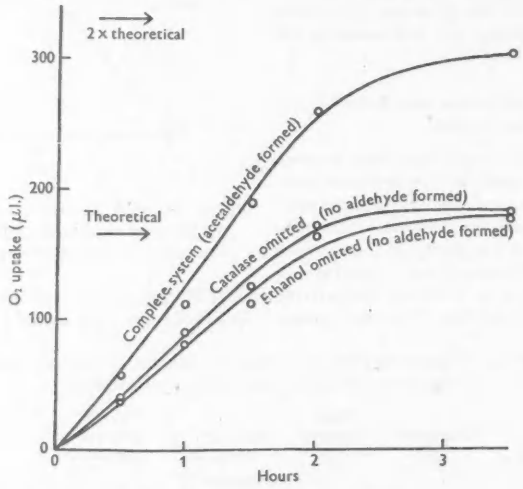


Fig. 3. Coupled oxidation of ethanol. Warburg manometers contained xanthine oxidase 8 mg., hypoxanthine 1 mg., ethanol 0·1 ml., crystalline bacterial catalase 0·8 mg., M/20 phosphate pH 7·2, total vol. 3 ml. Temp. 30°, gas phase air.

Once the molecular weight and *Kat.-f.* of the pure enzyme are known, it is possible to calculate the 'Turnover Number', defined by Warburg & Christian (1933) as the number of molecules of substrate decomposed by one molecule in 1 min. The relation between Turnover no. and *Kat.-f.* is obtained as follows. The initial velocity is obtained from the velocity constant k_0 by the first-order reaction equation $\left(\frac{-dS}{dt}\right)_{t=0} = 2{\cdot}303\ k_0 S_0$. By definition, $Kat.\text{-}f. = \frac{k_0}{\text{g. catalase/50 ml.}} = 20\, k_0/EM$, where S_0 and S are the concentrations of hydrogen peroxide at 0 and t min., and E is the concentration of catalase (all in mol./l.), and M is the molecular weight of catalase.

Hence,

$$\text{Turnover no.} = \frac{1}{E}\left(\frac{-dS}{dt}\right)_{t=0} = Kat.\text{-}f. \times 0{\cdot}115\, MS_0.$$

For bacterial catalase of mol. wt. = 232,000 and $Kat.\text{-}f.$ = 95,000, under the test conditions (0·0075 M-H_2O_2 at 0°), this gives a Turnover no. of 19×10^6, the highest recorded for any enzyme.

By similar calculations, the relation between $Kat.\text{-}f.$ and the Q_{O_2} (μl. O_2 evolved/mg. of enzyme/hr.) is given by

$$Q_{O_2} = Kat.\text{-}f. \times 77{,}500\, S_0.$$

Under the test conditions, bacterial catalase has a Q_{O_2} of 55×10^6. (The activity of catalase is usually measured at low hydrogen peroxide concentrations, when the percentage of total enzyme combined with substrate is small and proportional to the substrate concentration, so that the reaction is first order. Under these conditions k and $Kat.\text{-}f.$ are independent of the initial substrate concentration, but the Turnover no. and Q_{O_2} are directly proportional to the hydrogen peroxide concentration, which should always be stated.)

Coupled oxidation of alcohols

Keilin & Hartree (1936, 1945*b*) have shown that if catalase and ethanol are added to any enzyme system which produces hydrogen peroxide, the latter is used by the catalase to bring about a 'coupled oxidation' of the ethanol to acetaldehyde. In other words, under these special conditions (continuous slow supply of hydrogen peroxide at a very low concentration), the catalase acts as a peroxidase.

Fig. 3 shows that bacterial catalase behaves exactly like liver catalase in this respect. Xanthine oxidase-hypoxanthine was used as the source of hydrogen peroxide; the coupled oxidation is shown by a doubling of the theoretical oxygen uptake and the formation of acetaldehyde, recognized by its smell and the brown coloration imparted to the potassium hydroxide papers in the manometer cups. Acetaldehyde is only formed when catalase, ethanol, xanthine oxidase and hypoxanthine are all present.

DISCUSSION

The objects of this work were twofold: to investigate the use of lysozyme for the liberation of enzymes from the bacterial cell, and to apply this technique to the isolation of bacterial catalase.

Our present results indicate that the lysozyme technique is an excellent one, though not entirely without drawbacks. It is, of course, only applicable to lysozyme-sensitive micro-organisms. The viscous substances released from the bacteria by lysozyme (or possibly formed by its action) are a decided hindrance in the initial stages of enzyme purification. Nevertheless, the simplicity of the technique, the fact that it is unlikely to destroy labile enzymes, and its ready applicability on either a large or a small scale, should make it a useful tool in studying the intracellular components of bacteria.

The isolation of the catalase of *M. lysodeikticus* was undertaken partly in the hope that interesting differences might be revealed between the bacterial enzyme and the catalases of mammalian tissues. In fact, the differences observed are less striking than the similarities. The prosthetic group is the same as that of mammalian catalases, the molecular weight is the same, and there are the same number of haematin groups in the molecule. The main difference is that the catalytic activity of the bacterial enzyme is considerably higher than that of mammalian catalases; this difference must be attributed to the protein component of the enzyme.

The protein components of sheep, ox, horse and human catalases have all been shown to be different by immunological methods (Tria, 1939; Campbell & Fourt, 1939; Bonnichsen, 1947), and there is little doubt that the protein of bacterial catalase is different again. All these proteins, however, when combined with haematin, have the common property of catalyzing specifically the decomposition of hydrogen peroxide. It seems reasonable to suppose that this common property is related to some common structural element of the molecule—possibly some particular grouping of certain amino-acids—on which the catalytic activity depends. A careful study of the same enzyme isolated from several different sources might throw considerable light on the nature of enzymic catalysis. Catalase, which has now been isolated pure from so many different sources, should be a suitable enzyme to choose for such an investigation.

Another point of interest arising from this work is the remarkably high concentration of catalase found in *M. lysodeikticus*. This can be calculated from the $Kat.\text{-}f.$ values of the pure enzyme (95,000) and the lyzed bacteria (800–1800 in different batches), giving a catalase content for this organism of 1–2 % of its dry weight. This is about ten times the catalase content of human red blood corpuscles (Herbert & Pinsent, 1948). It can also be calculated that a single bacterial cell (dry weight taken as $2{\cdot}5 \times 10^{-13}$ g.) contains $10\text{–}20 \times 10^3$ enzyme molecules.

Such calculations raise the question of the function of catalase in *M. lysodeikticus*. The above concentrations of catalase would enable each bacterial cell to decompose *c.* 35–70 times its own weight of hydrogen peroxide/min. (at 0° and hydrogen peroxide concentration of 0·01M). Such large amounts of peroxide could scarcely be formed as a result of cell metabolism, and it is difficult to devise any teleological explanation for this high enzyme concentration unless, as the discovery of 'coupled

oxidation' by Keilin & Hartree (1936, 1945*b*) suggests, catalase has other functions besides the simple decomposition of hydrogen peroxide.

SUMMARY

1. The use of lysozyme in the study of intracellular bacterial enzymes and some phenomena noticed when lysozyme acts on concentrated bacterial suspensions are discussed.

2. Using lysozyme to liberate the enzyme from the bacterial cell, catalase has been isolated from *Micrococcus lysodeikticus* in crystalline form.

3. Bacterial catalase is in many respects similar to the catalases of animal tissues. The crystalline enzyme contains *c.* 1·1 % of haematin, identical with ordinary blood haematin.

4. It differs from liver catalases, but resembles erythrocyte catalases, in containing no verdohaematin.

5. Haematin content and ultracentrifugal data indicate a molecular weight of *c.* 230,000, with four haematin groups/molecule.

6. The catalytic activity is considerably higher than that of mammalian tissue catalases; the difference is to be attributed to the protein component of the enzyme.

Our thanks are due to Dr D. W. Henderson, Chief Superintendent of the Microbiological Research Department, Porton, and his staff, for their invaluable assistance in growing the large quantities of bacteria necessary for this work.

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Crystalline Human Erythrocyte Catalase

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The enzyme catalase has been isolated in crystalline form from ox liver (Sumner & Dounce, 1937), horse liver (Agner, 1942), lamb liver (Dounce, 1942), ox erythrocytes (Laskowski & Sumner, 1941), human and horse erythrocytes (Bonnichsen, 1947). In the preceding paper (Herbert & Pinsent, 1948) we describe the isolation of crystalline catalase from a bacterium, *Micrococcus lysodeikticus*.

With the exception of human-blood catalase, all these enzymes have been reported to contain about 0·097 % Fe or 1·1 % Fe-porphyrin, corresponding to four iron-porphyrin groups in a molecule of molecular weight 230,000; in bacterial and erythrocyte catalases all four iron-porphyrin groups are haematin, while in liver catalases a varying proportion may be verdohaematin or a related compound. This similarity between the catalases of three mammalian and one bacterial species is remarkable, and the reported exception of human-blood catalase is unexpected. This enzyme was crystallized by Bonnichsen (1947), who found it to contain only 0·077 % Fe and 0·83% haematin, and stated 'if we assume the same molecular weight as for the other catalases, this would mean that this catalase contains only three haematin groups'.

We have isolated crystalline catalase from human red cells by a new method. Contrary to Bonnichsen's findings, it contained 1·15 % haematin, corresponding to four haematin groups/molecule, if the molecular weight is assumed to be 220,000. Since no ultracentrifugal measurements have hitherto been reported for erythrocyte catalases, our enzyme was submitted to Dr R. Cecil, who found it to have a sedimentation constant of $11{\cdot}26 \times 10^{-13}$ (Cecil & Ogston, 1948), identical with the recorded values for liver catalases. Our preparation had a *Kat.-f.* of 63,000 and a ratio *Kat.-f.*/percentage of haematin of 54,400, which are similar to the values reported for other mammalian catalases (see Table 2, Herbert & Pinsent, 1948).

These results show human erythrocyte catalase to be essentially similar in catalytic activity and molecular weight to all other erythrocyte catalases, and to have the same number (four) of haematin groups/molecule. As our method of isolation evidently produces a purer enzyme than that of Bonnichsen (1947), and also gives higher yields, the details were thought to be worth placing on record.

EXPERIMENTAL

Methods. All methods used for determination of *Kat.-f.*, haematin content, etc., were exactly as described by Herbert & Pinsent (1948).

Isolation of crystalline human erythrocyte catalase

No particularly novel methods have been used in our isolation procedure. The first step consists in shaking the haemolyzed red cells with ethanol and chloroform (cf. Tsuchihashi, 1923); this removes all the haemoglobin, leaving the catalase in the supernatant fluid. The catalase is then absorbed on calcium phosphate gel. It may be eluted from the gel with 0·05N-ammonium hydroxide or disodium phosphate, but several washes with rather large volumes are necessary for complete elution, so that the final eluate is very dilute. Instead, we decompose the gel by shaking with potassium oxalate and centrifuging off the precipitated calcium oxalate. This gives quantitative recoveries in a small volume.

These two steps effect a hundredfold purification and give an enzyme approximately 20 % pure, from which pure, crystalline catalase may easily be obtained by careful fractionation with ammonium sulphate. Controlled salt fractionation has not much been used by previous workers on catalase, who have mostly preferred fractionation with organic solvents; our experience shows it to be an efficient as well as a simple technique.

The following are the details of a typical preparation:

Stage 1. The red cells were removed from citrated human blood by centrifuging, and washed twice on the centrifuge with 0·9 % NaCl; 6·84 l. of packed red cells were laked with an equal volume of distilled water, giving a solution containing 1690 g. total protein and 2·71 g. pure catalase; *Kat.-f.* = 101 (Note 1).

Stage 2. The laked red cells were treated with 0·44 vol. of a 3:1 ethanol-chloroform mixture, and stirred vigorously for 15 min. The sticky mass of precipitated haemoglobin was removed by straining through muslin and well pressed out. The rather turbid solution was filtered through a pad of 'Hyflo Super-Cel,' giving 14·6 l. of a clear, pale yellow filtrate showing no haemoglobin spectrum; *Kat.-f.* = 1620, yield 70 %.

Stage 3. The above filtrate was treated (Note 2) with 2 l. of a 1·5 % suspension of $Ca_3(PO_4)_2$ gel (Keilin & Hartree, 1938) and stood 1 hr. to allow the gel to settle. Most of the supernatant fluid could then be decanted off and the re-

mainder separated by centrifuging. The gel had adsorbed a considerable quantity of protein but very little catalase. A second addition of 30 g. of $Ca_3(PO_4)_2$ (Note 2) adsorbed almost all the catalase, and was collected by decantation followed by centrifuging. The packed $Ca_3(PO_4)_2$ precipitate was shaken vigorously with 600 ml. of 0·5 M-potassium oxalate in 0·5 M-NaH_2PO_4 (Note 3). The calcium oxalate was centrifuged off and washed twice with water, the washings being added to the first eluate. This gave 1620 ml. of a brown solution with a clearly visible catalase spectrum; *Kat.-f.* = 10,400, overall yield 56%.

Stage 4. Solid $(NH_4)_2SO_4$ was added to the above solution (30 g./100 ml.), and the greyish brown precipitate collected by centrifuging and dissolved in 100 ml. water; 20 ml. of N-acetate buffer pH 4·0 were then added and the solution allowed to stand 2 hr. (cf. Bonnichsen, 1947). A flocculent greyish precipitate formed and was removed by centrifuging, leaving a clear greenish brown liquor which was adjusted to about pH 5·6 with Na_2HPO_4; *Kat.-f.* = 21,800, overall yield 36%.

Stage 5. The catalase solution from stage 4 was treated with 4 M-$(NH_4)_2SO_4$, added drop by drop with good stirring. A small, greyish brown precipitate which appeared at an $(NH_4)_2SO_4$ conc. of 1·4 M (Note 4) was removed by centrifuging. Careful addition of $(NH_4)_2SO_4$ was continued until a faint turbidity appeared; on standing at room temperature this rapidly increased and developed a silken sheen, noticeable on stirring, like a suspension of bacteria. Microscopic examination showed it to consist of very fine needle-shaped crystals. After standing 1 hr. these were centrifuged down, and a further crop of crystals obtained from the mother liquor by adding a little more $(NH_4)_2SO_4$. The two crops of crystals were pooled and dissolved in 50 ml. of water; total 710 mg. or 26% overall yield on the red-cell haemolysate. The *Kat.-f.* was 50,200 and the haematin content 1·05%. After two recrystallizations by the same method, the *Kat.-f.* was raised to 63,000 and the haematin content to 1·15%.

Note 1. This batch of red cells had been kept for 13 days at 2° before using. In another preparation where the blood was used fresh, the washed, laked red cells had a *Kat.-f.* of 166. Both batches were pools from several donors.

Note 2. The quantity of calcium phosphate required varies from one preparation to another, and should be determined by a small-scale trial, treating, e.g. 10 ml. samples of enzyme with 1, 2, 3 and 4 ml. of gel suspension, centrifuging, and estimating the catalase left in the supernatant fluid.

Note 3. When $Ca_3(PO_4)_2$ is decomposed with oxalate the solution becomes strongly alkaline through the formation of K_3PO_4; hence NaH_2PO_4 is added to keep the pH neutral.

Note 4. The exact $(NH_4)_2SO_4$ concentration cannot be standardized, as it depends on many factors, including the catalase concentration; it is necessary to proceed empirically (cf. Herbert & Pinsent, 1948).

Properties of the crystalline enzyme

As already mentioned, the enzyme crystallizes from $(NH_4)_2SO_4$ in fine needles. Their refractive index is very similar to that of the mother liquor, which makes them difficult to photograph. On dialysis for many weeks against distilled water the enzyme crystallized in large irregular plates. It is more difficult to crystallize in this way than bacterial catalase; electrodialysis would probably be more efficient (cf. Bonnichsen, 1947). Solutions of the crystals in 0·05 M-phosphate pH 6·8 are reddish brown, and show absorption bands at 630, 544 and 505 mμ. (Hartridge reversion spectroscope). Treatment with pyridine, sodium hydroxide and sodium hyposulphite produces the characteristic spectrum of pyridine haemochromogen. On treating with acetone-hydrochloric acid (cf. Herbert & Pinsent, 1948) the prosthetic group is split off, and appears to consist entirely of haematin; no trace of biliverdin could be detected.

The purest sample obtained had a *Kat.-f.* of 63,000, and contained 1·15% haematin. If this is the pure enzyme, the molecular weight is $n \times 55{,}000$, where n is the number of haematin groups/molecule; for four haematin groups, the molecular weight would be 220,000. This sample was examined in the ultracentrifuge by Cecil & Ogston (1948) who found it to consist in the main of a homogeneous protein with a sedimentation constant of $11{\cdot}26 \times 10^{-13}$. This is in good agreement with the values of $11{\cdot}2 \times 10^{-13}$ for beef-liver catalase (Sumner & Gralén, 1938) and $11{\cdot}2 \times 10^{-13}$ for horse-liver catalase (Agner, 1938). This component accounted for 72% of the total refractive increment, and traces of another coloured protein were present in the ultracentrifuge photographs. Unfortunately some time had elapsed between the preparation of this sample and its examination in the ultracentrifuge, during which period the activity had decreased from the original value of *Kat.-f.* = 63,000 to 44,300, a decrease of 29%. We suspect, therefore, that the coloured impurity present was denatured catalase.

A rough estimate of the minimum molecular weight from the sedimentation constant is 176,000, assuming a spherical unhydrated molecule. If the molecule is not spherical, which is certainly the case (Sumner & Frampton, 1940), the molecular weight would be higher. It was unfortunately impracticable to perform measurements of partial specific volume and diffusion constant.

DISCUSSION

The results show that (within the limits of error of the various methods) human-blood catalase has essentially the same *Kat.-f.*, haematin content and molecular weight as other red-cell catalases, and like them, has four haematin groups/molecule. The reason for the discrepancy between our results and those of Bonnichsen (1947) is not clear. His preparations, however, had about the same *Kat.-f.*/percentage of haematin ratio as ours, although the absolute values of both were lower. The simplest explanation would be that his preparations were not pure; we found several recrystallizations necessary to obtain our purest enzyme. Our method of purification is simpler than any hitherto described, and should be applicable to the red cells of other species.

Crystalline erythrocyte catalase may now be considered a readily available enzyme.

It is interesting to calculate the catalase content of the human erythrocyte. This is obtained from the *Kat.-f.* of the pure enzyme (63,000) and of washed red cells, which in two different preparations was found to be 101 and 166. Hence catalase accounts for about 0·16–0·26 % of the total dry matter of the human red-blood corpuscle.

SUMMARY

1. The isolation of crystalline catalase from human red cells is described. The method is simple and the yield of crystalline enzyme is about 25 % of that present in the red cell.

2. The crystalline enzyme had a *Kat.-f.* of 63,000 and a haematin content of 1·15 %. The sedimentation constant was $11{\cdot}26 \times 10^{-13}$. The molecular weight was calculated to be 220,000 from the haematin content. There are four haematin groups/molecule; no verdohaematin is present.

Our thanks are due to Miss M. Mackay of the Medical Research Council's Blood Products Research Group, for kindly supplying us with large amounts of human red cells.

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Examination of Crystalline Catalases in the Ultracentrifuge

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Three samples of crystalline catalase were supplied by Dr Denis Herbert (Herbert & Pinsent, 1948 *a*, *b*). They were examined in a Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938). The solutions all contained approximately 0·5 % catalase in a buffer of composition sodium chloride 0·2 M, disodium hydrogen phosphate 0·05 M, sodium dihydrogen phosphate 0·05 M.

The speed was approximately 1015 rev./sec., and the wave length of light used 620–660 mμ. For the calculation of the sedimentation constants, the partial specific volume was taken as 0·73 ml./g. (Sumner & Gralén, 1938),

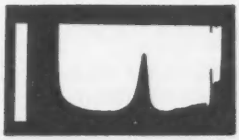

Fig. 1.

Fig. 2.

Fig. 3*a*.

Fig. 3*b*.

Fig. 1. Sample A I (bacterial catalase) approx. 25 min. after reaching full speed.

Fig. 2. Sample A II (bacterial catalase) approx. 25 min. after reaching full speed.

Fig. 3*a*. Erythrocyte catalase immediately on reaching full speed, showing heavier component.

Fig. 3*b*. Erythrocyte catalase approx. 25 min. after reaching full speed.

Note. The different appearance of the meniscus in Figs. 1 and 2 was due to the use of a layer of paraffin in these two runs.

Bacterial catalase (from Micrococcus lysodeikticus)

Sample A I. This had an activity corresponding to a *Kat.-f.* of 99,000 (Herbert, private communication). It gave a single homogeneous component with some heavier and lighter impurities (Fig. 1). The S_{20} (corr.) was $11{\cdot}0 \times 10^{-13}$, and integration of the boundary gave 85% of the initial refracting material.

Sample A II. This was another preparation of slightly lower activity, *Kat.-f.* = 90,000 (Herbert, private communication). It also gave a single homogeneous component with heavier and lighter impurities (Fig. 2), but integration gave only 70% of the initial refracting material. The S_{20} (corr.) was again $11{\cdot}0 \times 10^{-13}$.

Human erythrocyte catalase

A single sample was examined. The *Kat.-f.* when first prepared was 63,000, but during keeping it dropped to 44,300 (Herbert, private communication), suggesting that some denaturation had taken place. The sedimentation diagram was similar to that of the two previous samples except for a larger proportion of the heavier impurity, possibly denatured catalase. This impurity can be seen clearly in an early photograph (Fig. 3*a*), but it dispersed as the run proceeded (Fig. 3*b*) suggesting heterogeneity. Some of the pigment was seen to be associated with it. The S_{20} (corr.) of the main component was $11{\cdot}2 \times 10^{-13}$.

CONCLUSION AND SUMMARY

1. The ultracentrifugal data suggest that, while none of the preparations is fully homogeneous, there is in each case a main component which is associated with most of the pigment.

2. The sedimentation constants of this component in preparations from *Micrococcus lysodeikticus* and from human blood are similar (11·0 and $11{\cdot}2 \times 10^{-13}$ respectively), though the absolute difference is probably outside the experimental error.

3. The value of $11{\cdot}2 \times 10^{-13}$ for the sedimentation constant of human-blood catalase agrees with Agner's (1938) value of $11{\cdot}2 \times 10^{-13}$ for horse-liver catalase and with Sumner & Gralén's (1938) value of $11{\cdot}3 \times 10^{-13}$ for cow-liver catalase.

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Studies in Detoxication

17. THE FATE OF CATECHOL IN THE RABBIT AND THE CHARACTERIZATION OF CATECHOL MONOGLUCURONIDE

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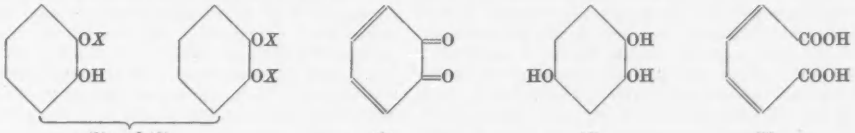
The main object of this investigation was to find out if conjugation of catechol occurs *in vivo* on one or both of its hydroxyl groups. The study of the fate of catechol was also necessary because it had a bearing on other problems being studied in this laboratory, namely, the metabolism of adrenaline (cf. Dodgson, Garton & Williams, 1947) and of benzene (cf. Porteous, Smith & Williams, 1947).

Despite the importance of catechol compounds in biochemistry, no significant study of the fate of catechol in the intact animal has been made other than that of Baumann (1876) who showed that administration of catechol to dogs caused an increased output of ethereal sulphate. However, numerous studies have been carried out *in vitro* of the formation and oxidation of catechol by enzymes of the tyrosinase type (see review by Nelson & Dawson, 1944).

From a consideration of the structure of catechol and of the results of enzyme studies, it is clear that it may form in the body (1) an ethereal mono- or disulphate or both, (2) a mono- or di-glucuronide or both, (3) *o*-benzoquinone by oxidation, (4) hydroxyquinol (1:2:4-trihydroxybenzene) and its conju-

gates, by oxidation, and (5) muconic acid by ring fission as suggested by Drummond & Finar (1938). These possibilities are formulated as follows:



(1) and (2) (3) (4) (5)

where X = an ethereal sulphate or glucuronic acid group.

Evidence will be presented to show that the metabolites excreted are mainly the monoglucuronide and monosulphate of catechol together with small amounts of conjugated hydroxyquinol.

EXPERIMENTAL

Animals. Giant chinchilla rabbits were used and fed on a diet of 50 g. Lever's cubes and water daily unless otherwise stated.

The effect of catechol on rabbits. Catechol is a relatively toxic substance. For cats and rats an oral dose of 50 mg./kg. is lethal (Dietering, 1938), whereas for rabbits the lethal oral dose is in the region of 200 mg./kg. (Masing, 1882; Brieger, 1879). In our experiments catechol (British Drug Houses Ltd., m.p. 103°), dissolved in water, was fed by stomach tube. We found that in five rabbits 100 mg./kg. had no apparent effect; at 175 mg./kg. slight toxic effects were apparent; at 200 mg./kg. the rabbits showed immediate signs of distress such as rapid breathing and twitching, with occasional paralysis of the limbs and squeaking. After about 3 hr. the animals appeared to be normal. One experiment was carried out using a dose of 250 mg./kg. which was fatal within 24 hr.

Quantitative results

Ethereal sulphate and glucuronic acid outputs. Ethereal sulphate excretion was determined by the method of Williams (1938) and glucuronic acid excretion according to Hanson, Mills & Williams (1944). The results are given in Table 1 and Fig. 1 illustrates a single experiment.

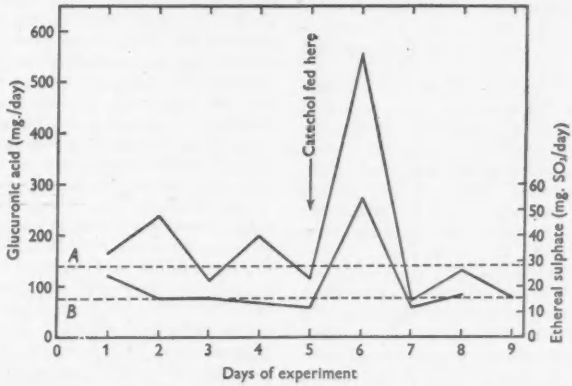


Fig. 1. The effect of feeding catechol (300 mg.) on the glucuronic acid and ethereal sulphate output of rabbit no. 113 (3 kg. wt.). Upper curve, glucuronic acid; lower curve, ethereal sulphate. Dotted lines show average normal values for (*A*) glucuronic acid, and (*B*) ethereal sulphate.

Table 1. *The glucuronic acid and ethereal sulphate excretion of rabbits receiving catechol orally*

Rabbit no.	Weight (kg.)	Dose (mg.)	Normal values of: Ethereal sulphate (mg./day)	Normal values of: Glucuronic acid (mg./day)	Extra ethereal sulphate (mg.)	Extra glucuronic acid (mg.)	% of dose excreted: As sulphate	% of dose excreted: As glucuronide
97* ♂	2·6	262	28·5	—	31·5	—	16·5	—
98* ♂	3·1	322	28·5	—	54·2	—	18·9	—
113† ♀	3·0	300	15·3	141	38·8	412	17·7	77·9
115† ♀	2·6	260	—	171	—	266	—	60·7
117† ♀	2·7	270	—	117	—	341	—	71·6
						Average	17·7	70

* Diet, 50 g. Lever's cubes + 100 g. cabbage per diem.
† Diet, 50 g. Lever's cubes + water *ad lib.* per diem.

Table 1 shows that about 17–18% of the catechol fed is excreted as a sulphate and about 70% as a glucuronide, assuming that monoconjugates are excreted. Furthermore, about 87–88% of the catechol fed can be accounted for by these conjugates excreted within 24 hr. of dosing. In the case of rabbit 113, it had been possible to carry out both estimations simultaneously and here the sum of the ethereal sulphate and glucuronide output is 95%. These results indicate that the main bulk of the catechol fed is excreted conjugated. A small amount of free catechol is also excreted which we assess at no more than 5%, for we isolated about 2% of the dose as derivatives of catechol (see below).

The isolation of catechol metabolites

(*a*) *Catechol urine.* Rabbits receiving oral doses of catechol excrete a slightly alkaline urine, which is normal in colour but darkens on standing. It gives the green colour characteristic of free catechol with $FeCl_3$, but does not reduce Fehling's or Benedict's reagents. It gives a strongly positive naphthoresorcinol reaction.

(*b*) *Detection of free catechol.* Urine (550 ml. in 24 hr.) from three animals after the feeding of a total of 0·68 g. of catechol was acidified with a few drops of conc. HCl, and extracted with ether for 1 hr. in a continuous extractor. The ether was removed at 20° under reduced pressure leaving 300 mg. of a brownish gum. This was dissolved in a few ml. of 40% (w/v) NaOH and benzoylated in the usual manner. Repeated recrystallization of the product from 95% ethanol yielded 10 mg. (0·7% of dose) of catechol dibenzoate, m.p. 83°, or 84·5° mixed with synthetic material (m.p. 85°).

(*c*) *Isolation and characterization of catechol monoglucuronide.* (i) *Preparation of the glucuronide gum.* A total of 7·59 g. of catechol was fed to 12 rabbits and a 24 hr. urine (1750 ml.) collected. The urine was clarified by centrifuging and made faintly acid (grey with congo red paper) by adding glacial acetic acid. Saturated lead acetate solution (350 ml.) was then added and the precipitate filtered off. The filtrate was made faintly alkaline with a little ammonia (sp.gr. 0·88) and treated with 200 ml. saturated basic lead acetate solution. The white precipitate was filtered off, washed well with cold water, made into a thin cream with water and the lead was removed completely with H_2S. The filtrate from the lead sulphide was taken to dryness *in vacuo* at 45–50°, and there remained a brown gum (12·5 g.) which showed a very slight reducing action towards Benedict's reagent and contained free catechol as indicated by its reaction with $FeCl_3$. The gum was taken up in absolute ethanol and on standing overnight a sludge of inorganic matter separated. This was removed by filtration and then the ethanolic solution was reduced to 30 ml. *in vacuo*. This solution was now poured into 300 ml. ether. Most of the glucuronide remained in solution but about 2 g. of gummy material containing much inorganic matter were thrown out. After removal of this, the ethanol-ether solution was concentrated *in vacuo* to a clear brown gum (10 g.). At this stage the purified gum gave a strong green colour with $FeCl_3$, a strong naphthoresorcinol reaction and reduced Benedict's reagent very slightly. Attempts to crystallize the glucuronide or to prepare crystalline salts with benzylamine, *o*- or *p*-toluidine or benzidine were unsuccessful.

(ii) *Removal of free catechol from the glucuronide gum.* The gum (6 g.) was dissolved in 75 ml. water and the solution was extracted with 3 × 100 ml. portions of ether. The extract was dried with anhydrous Na_2SO_4 and taken to dryness *in vacuo* at 20°. The semi-crystalline residue (730 mg.) gave intense colour reactions for catechol but gave no tests for hydroxyquinol (see below). It was dissolved in 12 ml. 2N-NaOH and immediately mixed with acetone (10 ml.) containing *p*-toluenesulphonyl chloride (1 g.). The mixture, which turned purple, was shaken vigorously for 15 min. and then poured into 30 ml. cold water. The white crystalline precipitate (300 mg., m.p. 159°) was collected and recrystallized from absolute ethanol. The product formed colourless needles and was identified as *catechol di-p-toluenesulphonate*, m.p. 161°. (Found: C, 57·8; H, 4·6; S, 14·95. $C_{20}H_{18}O_6S_2$ requires C, 57·4; H, 4·3; S, 15·3%.) This appears to be a new compound; an authentic specimen, m.p. 162–163°, and 161–162° when mixed with the material described above, has been prepared in another investigation (Porteous & Williams, unpublished). The yield of catechol ester corresponded to 1·8% of the catechol fed.

(iii) *Conversion of the glucuronide gum into the methyl ester of guaiacol triacetylglucuronide.* The solution after removal of catechol was taken to dryness and the residual gum (5·2 g.) was dissolved in 30 ml. absolute ethanol and treated with 250 ml. of a saturated ethereal solution of diazomethane. The mixture was kept at room temperature overnight. A slight precipitate of inorganic material was removed by filtration. The solution on evaporation yielded a pale yellow gum. Since the gum was acidic to litmus and incompletely soluble in ether, the treatment with diazomethane was repeated and on removal of the solvents a pale yellow, neutral, ether-soluble gum was obtained. This gum, which is presumably guaiacol glucuronide methyl ester, could not be induced to crystallize. It was therefore dissolved in 10 ml. pyridine followed by 15 ml. acetic anhydride and the mixture kept at room temperature overnight. On pouring into 50 ml. ice water a semi-solid gum was precipitated. This gum was now exhaustively extracted with chloroform yielding an orange-yellow solution which was washed twice with 2N-HCl, then twice with 10% (w/v) Na_2CO_3 solution and finally with water. The washed solution was now treated with charcoal, filtered and dried over $CaCl_2$. Removal of the solvent on the water bath left a clear yellow gum soluble in ethanol, acetone, ether and benzene but insoluble in water. Crystallization of the gum was achieved by dissolving in a minimum of acetone and then adding it to a large volume of warm water. Any cloudiness was dispersed by addition of a little acetone. After standing several days at room temperature, crystals contaminated with gum gradually appeared. The crystals were again recrystallized from a large volume of acetone-water (yield 2·83 g.). The compound was now easily recrystallized (charcoal) from aqueous ethanol and formed thick colourless needles, m.p. 107°. It gave the correct analysis for *o-methoxyphenyltriacetylglucuronide methyl ester* (*guaiacol triacetylglucuronide methyl ester*). (Found: C, 54·9; H, 5·5; OMe, 13·0. $C_{20}H_{24}O_{11}$ requires C, 54·5; H, 5·5; OMe, 14·1%.) $[\alpha]_D^{18°} = -49·3°$ ($c = 1$ in ethanol). It gave a positive naphthoresorcinol reaction for glucuronic acid on prolonged boiling (5 min.) with the reagents.

(iv) *Acid hydrolysis of the acetylated methyl ester. Isolation of guaiacol.* The methyl ester (0·75 g.) was heated under reflux for 6 hr. with 4N-HCl (50 ml.) and ethanol (5 ml.). The mixture, which smelt of guaiacol, was cooled and then

extracted with 3 × 75 ml. portions of ether. The combined ether extracts were extracted with small portions of 10% (w/v) NaOH solution (15 ml. in all). This alkaline extract which now contained the guaiacol was shaken for 10 min. with 5 ml. acetone containing 0·75 g. *p*-toluenesulphonyl chloride. The resulting brown solution was poured into 200 ml. water, but no solid appeared. The mixture was therefore heated at 60° for 1 hr. when a white precipitate appeared. After cooling, the precipitate (200 mg.) was collected and repeatedly recrystallized from ethanol. The product finally had m.p. 81–82°, and 82° mixed with authentic guaiacol *p*-toluenesulphonate, m.p. 82°. (Found: C, 60·3; H, 5·0; S, 11·65. Calc. for $C_{14}H_{14}O_5S$: C, 60·4; H, 5·1; S, 11·5%.) The residual solution, after ether extraction of the guaiacol, gave a positive naphthoresorcinol reaction and after neutralization reduced Fehling's and Benedict's reagents, thus showing the presence of glucuronic acid.

(*d*) *Detection of hydroxyquinol* (1:2:4-*trihydroxybenzene*) *in catechol urine.* Other work in this laboratory (Porteous & Williams, unpublished) had shown that benzene is converted in the rabbit to hydroxyquinol and we believe that this phenol is formed from benzene via catechol. It seemed likely therefore that on feeding catechol itself some hydroxyquinol might be produced.

Hydroxyquinol can be readily detected in the presence of catechol by two colour reactions.

(i) *The sulphuric acid test.* If small amounts of hydroxyquinol are gently warmed with conc. H_2SO_4, an intense red colour develops immediately. Phenol, catechol, quinol, pyrogallol and phloroglucinol do not give colours with H_2SO_4 under these conditions, whilst resorcinol gives a faint orange colour. By this test small amounts of hydroxyquinol can be detected readily in the presence of large amounts of catechol.

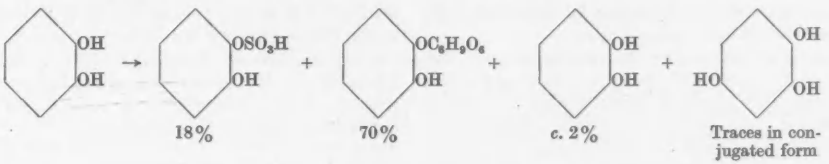
(ii) *The* 2:6-*dichloroquinone chloroimide test.* Traces of hydroxyquinol when added to 2–3 ml. of a saturated aqueous solution of 2:6-dichloroquinone chloroimide give a red colour which is intensified rapidly when the pH of the solution is raised to 8–9 by the addition of small amounts of $NaHCO_3$. This reagent also gives colours with phenol (blue) and catechol (blue-purple), but the hydroxyquinol colour is always dominant in a mixture at pH 8–9 (Porteous & Williams, unpublished).

Eleven rabbits were fed a total of 6·36 g. catechol (dose 200 mg./kg.) and urine (2 l.) collected over 24 hr. The urine was clarified by centrifuging and then made acid to litmus with a little dilute HCl. It was then continuously extracted for 5 hr. with peroxide-free ether. The extract contained catechol but no hydroxyquinol. From this we concluded that no hydroxyquinol was excreted in the free state. Ether was now expelled from the urine by gentle warming on the water bath, and the urine then was made N with respect to HCl and heated on a water bath for 0·5 hr. to hydrolyze ethereal sulphates but not glucuronides. The product was now subjected to continuous extraction with peroxide-free ether. Catechol and hydroxyquinol were detected in the extract up to 24 hr. after beginning the extraction. After 27 hr. extraction neither catechol nor hydroxyquinol was detected. The combined extracts were evaporated to dryness *in vacuo* and yielded 2·42 g. of semi-crystalline material. Catechol was removed by exhaustive extraction with dry benzene (4 × 40 ml.) in which hydroxyquinol is insoluble. Colour tests showed that the benzene extract contained no hydroxyquinol and on evaporation it yielded crystalline catechol. The residue (1·9 g.) after benzene extraction gave intense colorations in the H_2SO_4 and 2:6-dichloroquinone chloroimide tests for hydroxyquinol, but attempts to obtain crystalline derivatives were unsuccessful. The extracted urine was now warmed to expel ether and then made 3N with respect to HCl. The mixture was boiled under reflux for 1 hr. to hydrolyze glucuronides. After cooling, the urine was continuously extracted for 10 hr. with peroxide-free ether. The extract contained much catechol but no trace of hydroxyquinol. All extracts were also tested for pyrogallol without positive results.

It can, therefore, be concluded from these experiments that, in the rabbit, catechol is oxidized in small amounts to hydroxyquinol which appears in the ethereal sulphate, but not in the glucuronide, fraction of catechol urine.

DISCUSSION

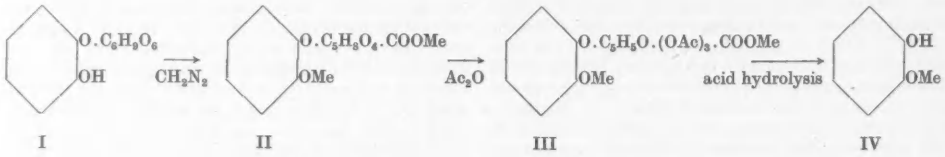
It is clear from the above that the metabolism of catechol can be represented as follows:



About 90% of catechol can be accounted for as catechol monoglucuronide, catechol monosulphuric acid, a small amount of free catechol and traces of conjugated hydroxyquinol, and these are excreted within 24 hr. after dosing (see Fig. 1).

The free catechol actually isolated as its crystalline di-*p*-toluenesulphonate was 1·8% of the dose and this must be considered as a minimum value owing to the losses inherent in the isolation of a small quantity of such a phenol. A figure of 5% of the dose would no doubt be nearer the true value for the free catechol excreted. The catechol glucuronide excreted has been proved to be a monoglucuronide. Methylation of the glucuronide (I) with diazomethane introduced two methyl groups thus indicating the presence of two acidic groups in the molecule, i.e. one free phenolic OH group and the carboxyl group of glucuronic acid. Methylation with diazomethane of the gummy catechol glucuronide did not yield a crystalline compound which could be characterized by melting point and optical rotation.

However, acetylation of the methylated glucuronide (II) yielded a crystalline product, viz. *o*-methoxyphenyl 2:3:4-triacetyl-β-D-glucuronide methyl ester (III). Hydrolysis of this ester produced guaiacol (IV) characterized as its *p*-toluenesulphonate. The isolation of guaiacol proves that the catechol glucuronide contains one free phenolic hydroxyl group and is therefore a monoglucuronide.



I II III IV

We have not provided direct proof that the ethereal sulphate excreted is a monosulphate but there is indirect evidence that this is so. Sulphuric esters of phenols are often extremely difficult to isolate from a complex fluid such as urine. Both the mono- and di-sulphuric esters of catechol have been prepared synthetically by Baumann (1878) who obtained the mono-ester as a crystalline potassium salt and the di-ester as an amorphous potassium salt. Neither of these esters has been isolated from biological sources (cf. Baumann & Herter, 1877–8). The mono-ester gives a violet colour with ferric chloride and such a colour can be obtained with acetone extracts of catechol urine.

It can be argued on theoretical grounds that the formation of a disulphuric ester from the mono-ester is unlikely, since catechol monosulphate is a phenol carrying a strongly acidic group in the *ortho* position. The situation is similar to that found in salicylic acid which does not form an ethereal sulphate in the rabbit (Williams, 1938). The reactivity of the free hydroxyl is suppressed for various reasons (*ortho* effect) by the adjacent strongly acidic group. Furthermore, Williams (1938) has shown that if, in an *o*-substituted phenol, the substituent group is a neutral one, e.g. the —OCH_3 group in guaiacol and the —CH_3 in *o*-cresol, then the extent of sulphate conjugation of that phenol is the same as found in phenol itself, i.e. *c.* 19% of the dose is excreted as sulphate. Assuming that catechol forms a monosulphate, the extent of its sulphate conjugation is 18% (see Table 1), in complete accord with the assumption that the second OH group is acting as a neutral group. There is also physico-chemical evidence which suggests that the reactivity of one hydroxyl group in catechol may be suppressed (e.g. Pauling (1945) on hydrogen bond formation in catechol; see also Ball & Chen (1933) and Florence & Schapira (1943) on dissociation constants). It is interesting to note that there are many instances known of compounds carrying two or more similar groups capable of conjugation *in vivo* but in which conjugation occurs only on one of these groups, e.g. orcinol (Sera, 1913, 1914), phloroglucinol (Sera, 1914), phenolphthalein (Di Somma, 1940), stilboestrol, hexoestrol and dienoestrol (Dodgson, Garton, Stubbs & Williams, 1948), etc.

Although the main fate of catechol in the rabbit involves conjugation only, it does undergo further oxidation to hydroxyquinol (1:2:4-trihydroxybenzene). Small amounts of conjugated hydroxyquinol were found in the urine, probably as an ethereal sulphate for it was only found in the ethereal fraction of the urine. From studies *in vitro* it has been shown that catechol is oxidized to hydroxyquinol by enzyme preparations containing catecholase (see review by Nelson & Dawson, 1944). Our results indicate that this oxidation also takes place in the intact animal. However, it is not clear from our experiments whether the hydroxyquinol arises by oxidation of free catechol or of a catechol conjugate. It is possible that it could arise by the oxidation of catechol monosulphate.

SUMMARY

1. The fate of catechol in the rabbit has been studied. At dose levels of 100 mg./kg., 70% of orally administered catechol is excreted in 24 hr. conjugated with glucuronic acid and 18% conjugated with sulphuric acid. A small amount, *c.* 2%, is excreted in the free state. Some catechol is also oxidized to hydroxyquinol which appears in the urine as an ethereal sulphate.

2. The catechol glucuronide has been proved to be a monoglucuronide, by conversion into crystalline *o*-methoxyphenyltriacetylglucuronide methyl ester which on hydrolysis yields guaiacol. Evidence is also presented to show that the ethereal sulphate of catechol formed in the rabbit is a monosulphate.

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Metabolism of Derivatives of Toluene

1. THE METABOLISM OF ACETOTOLUIDIDES IN THE RABBIT

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In an investigation of the metabolism of the acetotoluidides, Jaffe & Hilbert (1888) found that all three isomers were oxidized in the dog and rabbit. *p*-Acetotoluidide was shown to be converted almost quantitatively to *p*-acetamidobenzoic acid in both animals; similarly, *m*-acetamidobenzoic acid was isolated as a metabolite of *m*-acetotoluidide and it was suggested that an aminocresol might also be formed. *o*-Acetotoluidide gave rise in the dog to a compound claimed to be a methylbenzoxazolone. The constitution of this compound was not elucidated, but on treatment with ammonia in a sealed tube at 130–140° it gave a compound melting at 148–150° which appeared to be an aminocresol. Seven aminocresols were known to Jaffe & Hilbert, but it is now apparent that the isomer they obtained was in fact 2-amino-*m*-cresol which was not again described until 37 years later (Hodgson & Beard, 1925). The nature of the aminocresols formed as metabolites of acetotoluidides was of interest to us in view of the general rule concerning the position of entry of the hydroxyl group in biological hydroxylations which had been suggested by other investigations (e.g. Bray, Ryman & Thorpe, 1947; Bray, Lake, Neale, Thorpe & Wood, 1948). Consequently, we have carried out a further investigation of the fate of these compounds in the rabbit and included certain quantitative studies, the results of which are also reported here.

EXPERIMENTAL

Materials and methods

Materials. The acetotoluidides used in this study were prepared by the action of acetic anhydride upon the corresponding toluidines (Light and Co. Ltd.), the products being recrystallized from water. The reference compounds, 2-amino-5-hydroxytoluene (6-amino-*m*-cresol) and 5-amino-2-hydroxytoluene (5-amino-*o*-cresol), were prepared by the method of Noelting & Cohn (1884), which involves the reductive fission of azo compounds prepared from benzenediazonium chloride and the appropriate cresol. The azo compounds were dissolved in 2N-NaOH and reduced by means of $Na_2S_2O_4$. The properties of the compounds obtained agreed with those described in the literature.

Diet and feeding. The rabbits used were does of 2–3 kg. weight. A constant diet of rabbit pellets, with water *ad lib.*, was given throughout the study, as previously described (Bray *et al.* 1947). The acetotoluidides were administered by stomach tube as suspensions in water. The *p*-isomer showed no toxic properties at a dose level of 0·5 g./kg., but, since at this dose level the *m*-isomer was noticeably toxic and *o*-acetotoluidide slightly so, these two compounds were usually administered at a dose level of 0·25 g./kg.

Estimation of diazotizable material. This was carried out in acetotoluidide urines as collected, and after hydrolysis under the conditions described by Bratton & Marshall (1939). Compounds which might theoretically be included in the term 'diazotizable material' are aminocresols, with their glucuronides and ethereal sulphates, toluidines, and aminobenzoic acids. We found, however, that the two aminocresols

isolated as metabolites of *o*- and *m*-acetotoluidides gave no appreciable colour under the conditions of diazotization and coupling employed, and so made no significant contribution to the colour developed with either hydrolyzed or unhydrolyzed urine. We were not able to isolate glucuronides or ethereal sulphates of these compounds and their behaviour on diazotization and coupling is thus unknown. Since neither aminocresol is an *o*-aminophenol derivative, it would be expected that the colour given by the glucuronides or ethereal sulphates would be similar to that given by the parent compounds. However, these conjugates are found only in unhydrolyzed urine, and the amount of diazotizable material there is so small that it is in any case difficult to decide which compounds are responsible. In general, the colours obtained could be matched with relative ease against the colour developed from a standard solution of the corresponding aminobenzoic acid. The only diazotizable compounds, which are likely to make a contribution to the diazotizable material in hydrolyzed urine, are the toluidines and the aminobenzoic acids. The colours developed from these two types of compound cannot readily be matched, whereas the colours developed from hydrolyzed urine could in the majority of cases be readily compared with an aminobenzoic acid standard. This is indirect evidence for the absence of an appreciable amount of toluidines as is also the fact that there is reasonably good agreement between the results obtained from diazo estimations carried out on hydrolyzed urine and from estimations of ether-soluble acid, which determine the total acetamidobenzoic acids present less the amount excreted as ester glucuronide. While these facts do not provide final proof that unchanged acetotoluidides (or toluidines) are not excreted as such, they strongly suggest that this is the case. Moreover, we were never able to isolate any of these compounds from acetotoluidide urines.

Aminobenzoic acid solutions were used as standards in all diazo estimations. A much longer time was required for maximum colour development with the *o*-isomer than with the other compounds (Bray *et al.* 1948).

Estimation of reducing substances. The method used was that previously described (Bray, Neale & Thorpe, 1946*a*). The estimation was carried out only on unhydrolyzed urine, as aminocresols were found to reduce the Shaffer-Hartmann reagent and, in the case of *p*-acetotoluidide urines, we have found that the results obtained with urines passed after the administration of *p*-aminobenzoic acid were unreliable (Bray *et al.* 1948).

Estimation of ethereal sulphate. The method of Folin (1905–6) was used as before.

Estimation of ether soluble acid. The method used has already been described (Bray *et al.* 1946*a*). Under these conditions acetamidobenzoic acids may be quantitatively extracted and estimated. Since it was found that no phenolic material (i.e. aminocresols or their acetyl derivatives) was extracted during this procedure, the results of these estimations may be taken as giving the total oxidation to acetamidobenzoic acid less that excreted as ester glucuronide. The errors involved, owing to the fact that unacetylated aminobenzoic acids are not quantitatively extracted at the pH used, are small since the amounts excreted are very small.

RESULTS

Quantitative studies

Excretion of normal metabolites. On the diet used, it was found that the average daily excretion of ethereal sulphate ranged from 32 to 46 mg. SO_3; of ether-soluble acid (calculated as hippuric acid) from 709 to 913 mg., and of reducing material (calculated as glucuronic acid) from 224 to 307 mg.

Metabolism of p*-acetotoluidide.* The results of the estimations performed on *p*-acetotoluidide urines are summarized in Table 1. They strongly suggest that the oxidation to *p*-acetamidobenzoic acid is virtually complete, 96% of the dose being accounted for as

Table 1. *Metabolites of* p*-acetotoluidide in the rabbit*

		Percentage of dose excreted as				
Rabbit	Dose level (g./kg.)	Ether-soluble acid	Ester glucuronide	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0·5	84	5·2	—	92	Nil
157	0·25	—	—	—	—	Nil
	0·5	—	4·8	0·3	—	—
	0·5	—	7·8	0·8	—	—
	0·5	78	11·6	0·9	100	—
162	0·5	—	9·8	1·0	—	—
	0·5	—	7·0	0·9	—	—
163	0·25	—	8·4	—	—	Nil
	0·5	—	12·0	1·0	—	—
	0·5	—	7·7	0·8	—	—
	0·5	80	12·8	1·3	100	—
165	0·5	77	6·3	0·4	83	—
	0·5	80	—	1·1	100	—
166	0·5	77	9·4	0·3	94	—
170	0·5	78	17·9	0·3	95	—
	0·5	—	20·8	0·9	100	—
Average	—	79	10·0	0·8	96	Nil

this compound by diazo estimation, and 88% as the sum of the ether-soluble acid (78%) and ester glucuronide (10%). *p*-Acetotoluidide causes no increase in ethereal-sulphate excretion, as we anticipated, since Hammerbacher (1884) found the same for *p*-toluidine in the dog, and we showed that *p*-aminobenzoic acid had no effect upon ethereal-sulphate excretion in the rabbit (Bray *et al.* 1948). The amount of deacetylation which occurs is very small.

estimation. Attempts to isolate this compound have been unsuccessful, possibly because of such instability. A similar result was observed in the case of sulphapyridine, where there is no possibility of the formation of an ester glucuronide, and yet unhydrolyzed sulphapyridine urine had a reducing value greater than normal. It is possible that this may have been due to either (*a*) the instability of the glucuronide of hydroxysulphapyridine, a metabolite of the drug (Bray, Neale & Thorpe, 1946*b*),

Table 2. *Metabolites of* m-*acetotoluidide in the rabbit*

		Percentage of dose excreted as			
Rabbit	Dose level (g./kg.)	Ether-soluble acid	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0·25	36	0·3	23	—
	0·25	34	Tr.	27	—
153	0·25	30	—	25	—
	0·25	34	—	—	—
154	0·25	31	—	22	—
	0·25	19	—	26	—
157	0·25	49	Tr.	28	9
	0·25	48	Tr.	26	—
	0·25	42	—	—	—
163	0·25	29	Tr.	25	10
	0·25	27	Tr.	30	—
	0·25	27	—	—	—
173	0·25	24	Tr.	18	10
	0·25	37	Tr.	27	—
	0·25	31	—	—	—
	0·25	27	—	—	—
Average	—	34	Tr.	25	10

Metabolism of m-*acetotoluidide.* Table 2 summarizes the results of estimations carried out on the urine of rabbits which had received *m*-acetotoluidide. Since it is concluded that the results obtained from the estimation of total diazotizable material are due entirely to *m*-aminobenzoic acid, 25% of the dose appears to be oxidized to this compound, a value agreeing reasonably well with that obtained from the estimation of ether-soluble acid (34%). The value obtained from the diazo estimation is probably the more accurate since it does not involve 'base-line' values. The results of the estimations of reducing material are difficult to interpret and are not included in Table 2. In ten experiments the percentage of *m*-acetotoluidide excreted, apparently conjugated as ester glucuronide, ranged from 10 to 25 (average 16%). It is unlikely that this is a true value, since the diazo values should indicate the total extent of oxidation to *m*-acetamidobenzoic acid, i.e. should approximate to ether-soluble acid plus ester glucuronide. Three reasons may be suggested for the high reducing values obtained, but we have been unable to obtain direct proof of any of them. (i) It is possible that the glucuronide of 5-amino-*m*-cresol may be unstable under the conditions used for which might break down to glucuronic acid and hydroxysulphapyridine, which is itself reducing, or (*b*) reducing properties of the glucuronide itself, though these may have been due to (*a*). It may be significant that we were not able to isolate this glucuronide, in order to determine whether these suggestions have any foundation in fact. (ii) The second reason, which may be suggested to account for the high reducing values, is that *m*-acetotoluidide might have some effect upon carbohydrate metabolism. A similar effect was observed in connexion with *o*-acetotoluidide (see p. 214), but we have been unable so far to study the problem further. (iii) 5-Amino-*o*-cresol reduces the Shaffer-Hartmann reagent, but, since we have been unable to detect the free compound in *m*-acetotoluidide urines, it seems likely that it is only excreted in conjugated form.

Metabolism of o-*acetotoluidide.* This study gave results qualitatively similar to those on the *m*-isomer. It may be concluded from the diazo estimations (Table 3) that 6% of the dose is oxidized to anthranilic acid. This agrees reasonably well with the results obtained from the determinations of ether-soluble acid, though the latter cannot be regarded as precise since 8% of a dose of 600 mg.

Table 3. *Metabolites of o-acetotoluidide in the rabbit*

Rabbit	Dose level (g./kg.)	Percentage of dose excreted as: Ether-soluble acid	'Free' amino compounds	'Total' amino compounds	Ethereal sulphate
121	0·25	3·6	7·2	6·4	28
	0·25	3·4	5·2	6·2	—
153	0·25	6·9	5·7	5·9	—
	0·25	6·8	5·0	6·5	—
154	0·25	18·7	4·7	6·3	—
	0·25	16·8	4·8	5·8	—
157	0·25	—	—	—	30
163	0·25	—	—	—	37
173	0·25	8·5	8·2	7·5	—
	0·25	7·1	5·5	6·9	—
	0·25	16·0	4·5	5·1	—
Average	—	7·5	5·6	6·3	32

corresponds to an amount of acid (58 mg. hippuric acid) much less than the variations which occur in the normal 'base-line' values. Indeed, the excretion of ether-soluble acid in the 24 hr. period after the administration of *m*-acetotoluidide was in some cases actually within the normal range. Estimations of reducing value carried out on unhydrolyzed *o*-acetotoluidide urine gave results corresponding to conjugation of 25–68 % (average 42%) of the dose as ester glucuronide. It seems unlikely, from the agreement between the results of the diazo estimations and determinations of ether-soluble acid, that the reducing value actually represents ester-glucuronide excretion, and the explanations suggested in the case of the *m*-isomer may apply here also. Free 6-amino-*m*-cresol was not detected in *o*-acetotoluidide urines. We were also unable to isolate a glucuronide in order to test the hypothesis based on its instability. The fact that in one rabbit the increased excretion of reducing material (approximately twice the normal value) persisted for 4 days after dosing is in accordance with the suggestion that the high reducing values observed are due to some more general influence upon metabolism, e.g. of carbohydrate. In other rabbits the excretion of reducing material was restored to normal during the second 24 hr. We were unable to determine the nature of the reducing material excreted. In none of the rabbits was it accompanied by ketonuria, nor was it fermentable by yeast. We were unable to isolate a crystalline phenylosazone. There was little difference between the diazo results obtained with unhydrolyzed and hydrolyzed urine. The percentage of the dose excreted unacetylated was greater with *o*-acetotoluidide than with the other isomers. When anthranilic acid itself is administered, no acetylation appears to occur (Bray *et al.* 1948). A considerable percentage of *o*-acetotoluidide is hydroxylated and excreted conjugated with sulphate. This is in agreement with Hammerbacher's (1884) observation that *o*-toluidine caused an increase in excretion of ethereal sulphate in the dog.

Qualitative studies

o-Acetotoluidide. The urine of rabbits which had received this compound (1 g. each) was collected for 24 hr. after dosage, acidified with conc. HCl (0·1 vol.) and continuously extracted with ether for 24 hr. In this way a small amount of syrupy material (1 g. from urine of five rabbits) was obtained. This gave a slightly positive diazo reaction, which was much more intense after acid hydrolysis. From the syrup a small amount (10 mg.) of crystalline material (m.p. 184°) was isolated, and shown to be identical with authentic acetylanthranilic acid (m.p. 185°).

The urine was then acidified further with 0·1 vol. conc. HCl and hydrolyzed by boiling for 1 hr. The pH was adjusted to 8 and the urine continuously extracted with ether for 24 hr. A dark syrup was obtained which gave an intense red-purple colour with ferric chloride. Purification by dissolving in water, treatment with charcoal and evaporation of the filtrate gave colourless needles, darkening in air. m.p. 178° alone or mixed with 6-amino-*m*-cresol (m.p. 178°). This compound gives, on diazotization and coupling with dimethyl-α-naphthylamine or *N*-(1-naphthyl)-ethylenediamine dihydrochloride, a transient blue colour, but on long standing a violet colour develops. These colours are feeble compared with those obtained from aminobenzoic acids and in solutions of 10–20 mg./100 ml. are almost negligible. The yield of 6-amino-*m*-cresol cannot be definitely stated. Most procedures for isolation yielded about 100 mg. from a 1 g. dose, but owing to the great sensitivity of the compound to oxidation by air this does not represent the entire yield. In view of its instability it is reasonable to suggest that it is actually excreted as its *N*-acetyl derivative, though

we could not prove this directly, owing to its feeble diazotization and poor coupling properties. No free aminocresol was detected and no other metabolite was isolated.

o-Acetotoluidide urine gave a strongly positive naphthoresorcinol reaction and attempts were made to isolate a glucuronide (cf. Bray *et al.* 1947). These yielded only syrups which could not be crystallized, but from which, after hydrolysis, 6-amino-*m*-cresol was isolated.

m-*Acetotoluidide.* The method used for the isolation of metabolites of this compound was essentially the same as with the *o*-isomer. Extraction of the acidified urine yielded crystalline *m*-acetamidobenzoic acid which was purified by treatment with charcoal and recrystallization from water. It melted at 248° alone or mixed with an authentic specimen (m.p. 248°); yield, 20% of the dose. Extraction of the hydrolyzed urine gave a crystalline product, which after recrystallization from benzene had m.p. 174° alone or mixed with synthetic 5-amino-*o*-cresol. Its identity was further confirmed by comparison of acetyl derivatives obtained by the action of acetic anhydride at ordinary temperature. These compounds melted at 178° alone or mixed together. Yield, 30% of the dose. As with the aminocresol from *o*-acetotoluidide, it is probable that the acetyl derivative is the compound actually excreted.

p-*Acetotoluidide.* Extraction of acidified unhydrolyzed urine enabled 70% of the dose to be recovered as crystalline *p*-acetamidobenzoic acid. No other metabolite was isolated.

DISCUSSION

It can be seen that the results obtained in this investigation confirm in general those of Jaffe & Hilbert (1888). In addition we were able to show the formation from *o*-acetotoluidide of anthranilic acid, of which only small part is a excreted acetylated. 5-Amino-*o*-cresol was isolated from hydrolyzed *m*-acetotoluidide urine. In the rabbit *o*-acetotoluidide gives rise chiefly to 6-amino-*m*-cresol, whereas it now appears that Jaffe & Hilbert found 2-amino-*m*-cresol to be the chief metabolite in the dog. It is of interest that we obtained oxidation products of both these types from *o*-aminobenzoic acid in the rabbit (Bray *et al.* 1948), but we were unable to detect the vicinally substituted compound here. The configuration of the three aminocresols obtained by us, or by Jaffe & Hilbert, are in accordance with the general rule that the entering hydroxyl group takes up a position ortho or para to the amino (or acetamido) group already present (see Bray *et al.* 1948).

The formation of benzoxazolone or its derivatives *in vivo* is of interest. To our knowledge only three such cases have been described, viz. from acetanilide and *o*-acetotoluidide (Jaffe & Hilbert, 1888), and from formanilide (Kleine, 1896–7). In all these studies the experimental animal was the dog. It would be of interest to determine whether any other animal is capable of ring formation of this type, since *o*-aminophenol compounds are common metabolites of amino compounds. There is a possibility that the benzoxazolone derivatives formed may be artifacts, since it is known that *o*-aminophenol (or its hydrochloride) and urea give rise to benzoxazolone when heated together at a high temperature (Sandmeyer, 1886; Williams, 1947). In all three instances where benzoxazolone was isolated the urine was first evaporated to dryness. Whether this treatment is drastic enough to deacetylate the *o*-aminophenol derivative and then condense it with urea appears doubtful, but this question is under investigation.

The quantitative results show an interesting gradation in metabolic behaviour, as can be seen in Table 4.

It is probable that the *N*-acetyl group in *p*-acetotoluidide remains intact throughout metabolism, since the degree of acetylation of the *p*-aminobenzoic acid excreted (at least 88%) is very much greater than when this acid is itself ingested (4–21%, Bray *et al.* 1948). It is not possible to say, with any certainty, whether this applies also to the other acetotoluidides, since other metabolites are also formed.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-acetotoluidides in the rabbit has been studied.

2. *o*-Acetotoluidide is partly oxidized to anthranilic acid (6%) and acetyl anthranilic acid (1%); a considerable part of the remainder appears to be converted to 6-amino-*m*-cresol, which has been isolated. This latter compound is probably excreted as its

Table 4. *Summary of metabolites of acetotoluidides in the rabbit*

	Average percentage of dose excreted as				
Isomer	Ether-soluble acid	Ester glucuronide	'Free' diazotizable material	'Total'* diazotizable material	Ethereal sulphate
ortho	8	—	5·6	6	32
meta	34	—	Tr.	25	10
para	79	10	0·8	96	Nil

* Assumed equivalent to total oxidation of CH_3 group.

N-acetyl derivative conjugated with glucuronic acid and sulphate. The extent of sulphate conjugation corresponds to 32% of the dose.

3. The metabolism of *m*-acetotoluidide is similar to that of the ortho isomer; 25% is oxidized to the corresponding *N*-acetamidobenzoic acid, and the only other metabolite detected was 5-amino-*o*-cresol which has been isolated. This is probably excreted as its *N*-acetyl derivative conjugated with glucuronic acid and sulphate; 10% of the dose is excreted as an ethereal sulphate.

4. *p*-Acetotoluidide is virtually completely oxidized to *p*-acetamidobenzoic acid, 10% being excreted as ester glucuronide. There is no conjugation with sulphuric acid.

5. *o*- and *m*-acetotoluidides cause an abnormally high excretion of reducing material in urine. The reason for this has not been determined.

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Studies in the Biochemistry of Micro-organisms

78. THE MOLECULAR CONSTITUTION OF MYCOPHENOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX

PART 2. POSSIBLE STRUCTURAL FORMULAE FOR MYCOPHENOLIC ACID

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Mycophenolic acid, $C_{17}H_{20}O_6$, was first named and isolated from cultures of *Penicillium stoloniferum* Thom by Alsberg & Black (1913), although Gosio (1896) had reported an unnamed crystalline metabolic product of '*P. glaucum*', to which he assigned the formula $C_9H_{10}O_3$, which is probably identical with mycophenolic acid. Clutterbuck, Oxford, Raistrick & Smith (1932) isolated mycophenolic acid, together with other phenolic compounds, from the culture filtrates of twelve species or strains in the *P. brevi-compactum* series, to which *P. stoloniferum* Thom belongs, and Clutterbuck & Raistrick (1933) reported an extensive chemical investigation of this substance.

Gosio (1896) demonstrated that his substance, $C_9H_{10}O_3$, suppresses the growth of the anthrax bacillus, so that it appears probable that mycophenolic acid, although of relatively low potency, was the first antibiotic of fungal origin to be obtained in crystalline form. More recently, Florey, Gilliver, Jennings & Sanders (1946) investigated its antibacterial action against a number of animal and plant pathogens and also against a number of saprophytic and pathogenic fungi. They showed that it is more effective against Gram positive than against Gram negative bacteria and that its activity against staphylococci varies greatly with the number of organisms used for the inoculum. This 'inoculum size' phenomenon was thoroughly investigated by Abraham (1945).

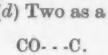
Clutterbuck & Raistrick (1933) established, *inter alia*, the following facts bearing on the constitution of mycophenolic acid:

(1) It is optically inactive.

(2) The six oxygen atoms are present in the molecule in the following forms: (*a*) One as a OCH_3 group. On demethylation, normycophenolic acid, $C_{16}H_{18}O_6$, is formed. (*b*) One as a phenolic OH group, re-

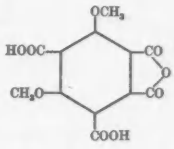
sponsible for the blue-violet $FeCl_3$ colour. (*c*) Two as a COOH group. (*d*) Two as a lactone ring,

(3) It contains the aliphatic grouping



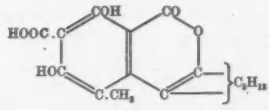
(4) On fusion with KOH 1:5-dihydroxy-3:4-dimethylbenzene is formed.

(5) Its monomethyl ether, on protracted oxidation with alkaline $KMnO_4$, gives the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene (I) in good yield.



(I)

On the basis of these facts, and on others for which the original paper should be consulted, the structure (II) for normycophenolic acid was advanced.



(II)

No evidence was available as to which of the two phenolic groups in (II) is methylated in mycophenolic acid itself.

We now present experimental evidence which necessitates modification and amplification of structure (II) and may be summarized as follows.

I. *Ozonization of mycophenolic acid, its monomethyl ether, and its monomethyl ether methyl ester*

Chloroform solutions of these three substances were ozonized, and, after decomposing the resulting ozonides with water, the following breakdown products were isolated.

(*a*) *From mycophenolic acid* ($C_{17}H_{20}O_6$, with 1 OCH_3 group): levulic acid, $CH_3.CO.CH_2.CH_2.COOH$; and an aldehyde $C_{12}H_{12}O_5$, containing 1 OCH_3 group, giving the same $FeCl_3$ reaction as the parent substance, and not extractable from chloroform with aqueous $NaHCO_3$.

(*b*) *From mycophenolic acid monomethyl ether* ($C_{18}H_{22}O_6$, with 2 OCH_3 groups): levulic acid; an aldehyde, $C_{13}H_{14}O_5$, containing 2 OCH_3 groups, which will be dealt with more fully later; and a small amount of the corresponding carboxylic acid, $C_{13}H_{14}O_6$.

(*c*) *From mycophenolic acid monomethyl ether methyl ester* ($C_{19}H_{24}O_6$, with 3 OCH_3 groups): methyl levulate, $CH_3.CO.CH_2.CH_2.COOCH_3$; and the same aldehyde $C_{13}H_{14}O_5$, containing 2 OCH_3 groups, as was obtained by ozonization of mycophenolic acid monomethyl ether.

These findings clearly prove that mycophenolic acid contains: (1) a hydroxyl group, directly attached to the nucleus, which can be methylated by diazomethane and is therefore phenolic in nature; and (2) a free carboxyl group in a side chain which gives rise to levulic acid on ozonolysis of mycophenolic acid or its monomethyl ether and to methyl levulate on similar treatment of its monomethyl ether methyl ester.

The free COOH group attached directly to the nucleus in structure (II) must, therefore, now be transferred to the end of a side chain of six or more carbon atoms including the C_5H_{12} residue, the nature of which was left undetermined by Clutterbuck & Raistrick (1933).

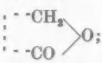
II. *Molecular constitution of the aldehyde*, $C_{13}H_{14}O_5$

The substance, $C_{13}H_{14}O_5$, obtained by the ozonolysis of both the monomethyl ether and the monomethyl ether methyl ester of mycophenolic acid, is a neutral substance and is a true aldehyde and not a ketone. It gives typical aldehyde reactions, is oxidized by alkaline iodine to a carboxylic acid, $C_{13}H_{14}O_6$, and on heating with acetic anhydride and anhydrous sodium acetate it gives a mono-enol acetate. The important conclusion follows that in the aldehyde, $C_{13}H_{14}O_5$, the CHO group is not attached *directly* to the nucleus but through an intervening $—CH_2—$ link, the acetylated product having the form $R.CH{:}CH.O.CO.CH_3$. This conclusion is confirmed by the fact that ozonolysis of the enol acetate yields an aldehyde, $C_{12}H_{12}O_5$, fission having occurred at the double linking.

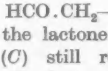
Of the thirteen carbon atoms present in the aldehyde, $C_{13}H_{14}O_5$, six are present in a benzene ring, since permanganate oxidation of mycophenolic acid monomethyl ether yields the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene, structure (I), two as OCH_3 groups and two as the group $—CH_2.CHO$, leaving three carbon atoms to be accounted for. It follows from the structure of the above anhydride (I) that *each* of these three carbon atoms must be attached *singly* to the benzene nucleus.

The nature of the groups in which each of these three carbon atoms is present may now be considered. The aldehyde $C_{13}H_{14}O_5$ (*A*) behaves as a neutral substance on titration with NaOH in the cold, but as a monobasic acid on heating. It is thus

probably a monolactone. Alkaline iodine converts it into a compound $C_{13}H_{14}O_6$ (*B*) which still retains two methoxy groups but has lost its aldehydic properties. It titrates in the cold as a monobasic acid and on heating as a dibasic acid. It is therefore a monobasic acid monolactone. The disodium salt of (*B*), on oxidation with cold neutral aqueous $KMnO_4$, yields a substance which, after sublimation in a high vacuum, has the empirical formula $C_{13}H_{12}O_7$ (*C*). This substance still retains both OCH_3 groups, and titrates sharply in the cold as a tribasic acid. These facts can be most simply explained by postulating that (*A*) contains the groups $HCO.CH_2$— and

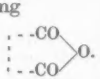


that in (*B*) the $HCO.CH_2$— group becomes $HOOC.CH_2$— and the lactone grouping remains unchanged; that (*C*) still retains the group $HOOC.CH_2$— while the lactone ring is opened in the sodium salt to



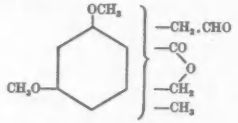
and oxidized and the ring reclosed on sublimation to give the anhydride ring

Thus we may conclude that two of the three carbon atoms in (*A*), whose nature is in doubt, are vicinal and in the form



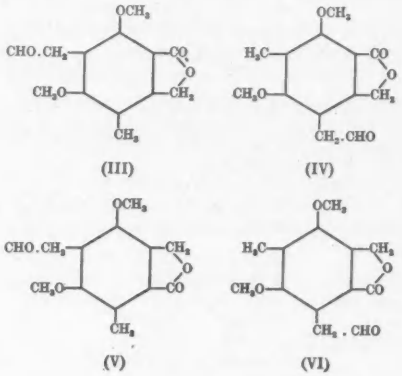
The third carbon atom is present as a CH_3 group, since the aldehyde $C_{13}H_{14}O_5$ (*A*) was shown by analysis to contain one carbon-methyl, $CH_3(C)$, group. Mycophenolic acid itself was shown to contain two carbon-methyl groups, the second one being the branched CH_3 group in the side chain in structures (IX) and (X).

Since the two methoxyl groups are attached directly to the benzene nucleus in the meta position to each other (Clutterbuck & Raistrick, 1933) the constitution of the $C_{13}H_{14}O_5$ aldehyde may therefore be written in the form



and since each of the four different substituent groups, two of them, viz.

vicinal, must be attached directly to the nucleus, there are only four possible isomers, (III), (IV), (V) and (VI).



(III) (IV)

(V) (VI)

Evidence as to which of these four structural formulae most probably represents the structure of the aldehyde $C_{13}H_{14}O_5$, and hence part of the structure of the monomethyl ether of mycophenolic acid from which it is derived by ozonization, is available from a number of sources, all of which lead to the same conclusion.

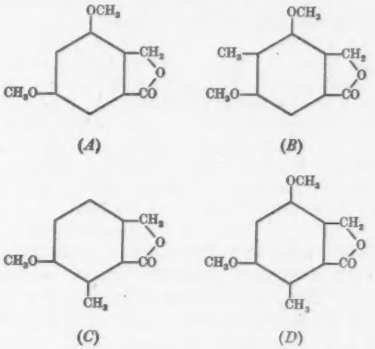
(*a*) On the addition of a suspension of 2:6-dichloroquinone chloroimide to a solution of normycophenolic acid in sodium borate buffer of pH 9·2, at which pH the phthalide ring will be opened and a COOH group liberated, a blue colour with a tinge of green is *rapidly* formed. The *rapid* production of a blue colour by a substituted benzene compound is indicative, according to Gibbs (1927), of a free position para to a phenolic group, though according to Davidson, Keane & Nolan (1943) the reaction is also positive if the position para to OH is occupied by COOH. Examination of structures (III), (IV), (V) and (VI), assuming the OCH_3 groups to be replaced by OH groups as in normycophenolic acid, shows that only (III) and (IV) satisfy the requirement of COOH para to OH.

(*b*) Clutterbuck & Raistrick (1933) showed that, on fusion with KOH, mycophenolic acid yields 1:5-dihydroxy-3:4-dimethylbenzene. Hence, if one ignores the possibility of migration of substituents during potash fusion, two of the three substituents, CH_3, CH_2OH and $CH_2.CHO$, likely to give rise to the two CH_3 groups in the fusion product, must be vicinal. This requirement also is satisfied only by (III) and (IV).

(*c*) One of the groups shown as methoxyl in (V) and (VI) is represented by a free (phenolic) OH in mycophenolic acid itself. As both methoxyl groups of (V) and (VI) are meta to the potential carboxyl group, the OH group of mycophenolic acid must also

be meta to the potential carboxyl if either (V) or (VI) is correct. This orientation would be unlikely to give rise to the intense *violet* coloration which mycophenolic acid gives with ferric chloride, whereas the possible *o*-OH group derivable from (III) and (IV) would be expected to give such a colour.

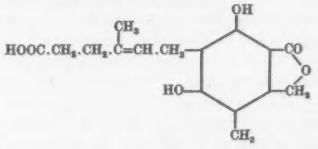
(*d*) Of the four known phthalides:



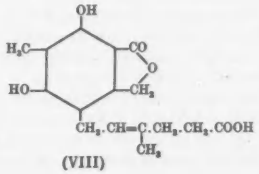
(*A*) (*B*) (*C*) (*D*)

(*A*) (Fritsch, 1897) and (*B*) (Charlesworth & Robinson, 1934) are readily oxidizable to the corresponding phthalic acid by means of alkaline permanganate, whereas (*C*) (Charlesworth, Rennie, Sinder & Yan, 1945) and (*D*) (Mittar, Sen & Paul, 1927; Charlesworth *et al.* 1945) are not so oxidized. It appears that the presence of a substituent methyl group ortho to the potential carboxyl of the phthalide grouping prevents oxidation. If this is the case it would rule out formulae (V) and (VI) as possibilities for the C_{13} aldehyde, since (V) has a methyl group and (VI) a substituted methyl group ortho to the potential carboxyl, yet the product, after oxidation of —CHO to —COOH was oxidized by permanganate to the phthalic acid.

The two most probable formulae for normycophenolic acid therefore become (VII), from (III), and (VIII), from (IV).



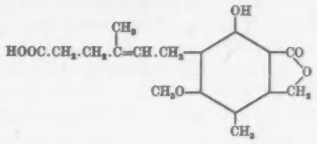
(VII)



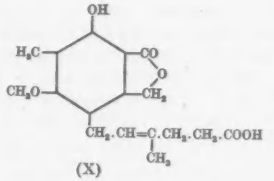
(VIII)

We have no experimental evidence capable of deciding between (VII) and (VIII), but we are inclined to favour (VII), since this structure contains a preformed CH_3 group oriented towards the two OH groups as in 1:5-dihydroxy-3:4-dimethylbenzene, the potash fusion product of mycophenolic acid.

Finally, there arises the question as to which of the two OH groups in (VII) or (VIII) is methylated in mycophenolic acid itself. We have stated previously that normycophenolic acid rapidly gives a blue colour with 2:6-dichloroquinone chloroimide. Under the same conditions mycophenolic acid gives only a very faint dirty yellow colour even on standing. We are aware of the dangers of interpreting a negative 2:6-dichloroquinone chloroimide reaction (see Calam, Clutterbuck, Oxford & Raistrick, 1947), but, with this reservation, the choice of the hydroxyl group to be represented as methylated in mycophenolic acid has fallen on that para to a potential carboxyl group. This choice is strengthened by the fact that an aqueous solution of mycophenolic acid gives with aqueous $FeCl_3$ a strong violet colour somewhat similar to, but admittedly bluer than, that given by salicylic acid. Hence we believe that the more probable structure for mycophenolic acid is (IX) and the less probable (X).



(IX)



(X)

It remains now to show that the experimental evidence obtained by Clutterbuck & Raistrick (1933) can be explained by the favoured constitution for mycophenolic acid. In most cases this is self-evident, but two points call for further discussion.

Titration values. On titration to phenolphthalein in water with aqueous NaOH in the cold, mycophenolic acid and its derivatives gave the following titration equivalents:

(*a*) Normycophenolic acid, 155·6. Theor. as a dibasic acid, 153.

(*b*) Mycophenolic acid, 169. Theor. as a dibasic acid, 160.

(*c*) Mycophenolic acid monomethyl ether, 332. Theor. as a monobasic acid, 334.

(*d*) Monoacetylmycophenolic acid, 361. Theor. as a monobasic acid, 362.

On heating the cold titrated solutions with excess of NaOH and back titrating, mycophenolic acid and its monomethyl ether became fully dibasic, while acetylmycophenolic acid became tribasic.

The interpretation of these findings appears to be as follows:

(1) Monoacetylmycophenolic acid is completely hydrolyzed with the formation of one molecule each of acetic and mycophenolic acids.

(2) Mycophenolic acid monomethyl ether. Since there is no free phenolic OH group in this compound either before or after hydrolysis, the ether being recovered unchanged from the acidified hydrolysis solution, it is clear that the free carboxyl group at the end of the long substituent chain titrates in the cold and that, on heating, the phthalide ring is opened and the COOH group thus liberated then titrates. The formation of a disilver salt,

$$C_{16}H_{22}O_5(COOAg)_2,$$

involving the addition of the elements of water confirms this view.

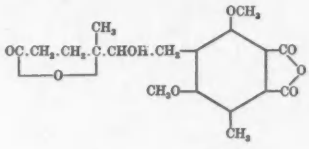
(3) Mycophenolic acid. Two explanations seem possible. (*a*) One equivalent of acid is due to the free terminal COOH group. The second (partial) equivalent in the cold is due to the partial, but almost complete, opening of the phthalide ring in the cold. This, however, is contrary to the usual behaviour of the phthalide grouping. (*b*) An alternative explanation is that the second (partial) equivalent in the cold is due to the acidity of the phenolic OH group. It must then be assumed that, on hot alkaline hydrolysis, the phthalide ring is opened, and that the resulting formation of a nuclear carboxyl group has suppressed the ionization of the phenolic OH group, so that the latter has no longer a titratable acidity. This view is confirmed by the fact that according to Alsberg & Black (1913) mycophenolic acid forms two barium salts having the empirical formulae $(C_{17}H_{19}O_6)_2Ba$ and $C_{17}H_{18}O_6Ba$. The latter dibasic salt clearly arises without the addition of the elements of water, and hence, inferentially, without the opening of the phthalide ring.

The constitution of the alkaline permanganate oxidation product of mycophenolic acid monomethyl ether (Clutterbuck & Raistrick, 1933, p. 659)

This substance, which was isolated in good yield, has the empirical formula $C_{16}H_{14}O_6(OCH_3)_2$. It has, therefore, the same number of carbon atoms as the ether from which it is formed according to the equation

$$C_{16}H_{16}O_4(OCH_3)_2 + 3O \rightarrow C_{16}H_{14}O_6(OCH_3)_2 + H_2O.$$

Since, unlike the parent ether, it does not immediately decolorize $KMnO_4$ in the cold, it probably arises by oxidation at the double bond to the glycol form (requiring one O), followed by lactonization on subsequent isolation in acid solution, coupled with oxidation of the phthalide to the phthalic anhydride (requiring two O), the product having the constitution (XI) from structure (IX) or the corresponding structure derived from (X):



(XI)

It was found that the oxidation product, when dissolved in ethanol, titrated in the cold as a monobasic acid, but after alkaline hydrolysis as a tribasic acid. A product of structure (XI), when dissolved in ethanol, should yield the mono-acid mono-ester derived from the acid anhydride ring. This would then titrate in the cold as a monobasic acid, whereas two new acidic groups would be revealed on alkaline hydrolysis, one by hydrolysis of the ester grouping and the other by the opening of the lactone ring. This would also explain the fact that the substance was recovered unchanged after acidifying the hydrolysis solution.

EXPERIMENTAL

A. *Action of ozone on mycophenolic acid and its derivatives*

In this series of experiments ozonized O_2 was passed through a chloroform solution of the substance until a sample of the solution no longer decolorized a dilute solution of Br_2 in chloroform. The chloroform solution of the ozonide was well shaken with its own volume of water, warmed to *c.* 50° and again thoroughly shaken to decompose the ozonide. After cooling, the water fraction was separated from the chloroform fraction and the two were worked up separately.

I. *Action of ozone on mycophenolic acid.* Mycophenolic acid (0·5 g.) in chloroform (25 ml.) was ozonized for 2·5 hr.

Water fraction. The solution was treated with $NaHCO_3$ (0·5 g.) and continuously extracted with ether. On removal of the solvent only a trace of whitish residue remained. The extraction was continued after acidification with 2N-H_2SO_4. The oil (0·18 g.) thus obtained was treated with 50 ml. of Brady's reagent (0·3% 2:4-dinitrophenylhydrazine in 2N-HCl). An orange-yellow crystalline precipitate soon formed, m.p. 203–205°, raised to 205° after recrystallization from methanol, and not depressed on admixture with authentic levulic acid 2:4-dinitrophenylhydrazone, m.p. 205–206°. The substance is acid to litmus, and is readily soluble in aqueous $NaHCO_3$ to a golden yellow solution from which it separates on acidification as an orange flocculent precipitate.

Chloroform fraction. The chloroform solution was washed with aqueous $NaHCO_3$. The acids were liberated by acidification with 2N-H_2SO_4 and extracted with ether, yielding 0·06 g. of colourless crystalline solid which was not further examined. The neutral fraction, after removal of chloroform, remained as a mixture (0·21 g.) of crystals and oil. It was washed with a little ether to remove the oil, and the remaining crystals, after recrystallization first from ether and then from water, gave 0·07 g. of short colourless rods, m.p. 152–153°. (Found: C, 61·23, 61·14; H, 5·12, 5·40; OCH_3, 12·7, 13·3. $C_{11}H_8O_4(OCH_3)$ requires C, 61·01; H, 5·12; OCH_3, 13·1%.) The substance gave a strong aldehyde reaction (Schiff), and gave in water and in ethanol with $FeCl_3$ the same colour reactions as mycophenolic acid. It dissolved readily in cold 2N-NaOH giving an immediate yellow colour which became somewhat darker on standing. The substance is therefore an aldehyde, $C_{12}H_{12}O_5$, with one methoxyl group, and evidently the grouping, a phenolic OH group, responsible for the $FeCl_3$ reaction with mycophenolic acid, persists in its ozonolysis product.

II. *Action of ozone on mycophenolic acid monomethyl ether.* Mycophenolic acid monomethyl ether (1·0 g., m.p. 112°; see Clutterbuck & Raistrick, 1933, p. 658) in chloroform (50 ml.) was ozonized for 2·25 hr. A total of 5·30 g. of the ether was ozonized.

Water fraction. The combined water fractions were treated with an excess of Brady's reagent giving an orange crystalline precipitate, 1·16 g., m.p. 201–203° of levulic acid 2:4-dinitrophenylhydrazone. A sample recrystallized from methanol in orange needles, m.p. 204–205°, was analyzed. (Found: C, 44·38; H, 4·63; N, 19·05. $C_{11}H_{12}O_6N_4$, i.e. the dinitrophenylhydrazone of $C_5H_8O_3$, requires C, 44·60; H, 4·08; N, 18·9%.)

Chloroform fraction. The combined chloroform extracts were washed twice with 50 ml. aqueous $NaHCO_3$, and this extract on acidification yielded 0·39 g. of almost colourless crystals with some oil. On recrystallization from chloroform—light petroleum colourless needles were obtained, m.p. 153°, identical with the alkaline iodine oxidation product of the aldehyde $C_{13}H_{14}O_5$. (Found: C, 59·05, 59·20; H, 5·17, 5·10. $C_{13}H_{14}O_6$ requires C, 58·64; H, 5·30%.)

The bicarbonate-extracted chloroform solution, on removal of the solvent, yielded an oil which soon crystallized. On recrystallization from water a colourless crystalline solid, 2·05 g., of constant m.p. 112° was obtained. Concentration of the aqueous mother liquors gave a further 0·4 g., m.p. 107°. The substance gives a strong Schiff reaction and is the same aldehyde, $C_{13}H_{14}O_5$, m.p. 112° as was obtained by the ozonization of mycophenolic acid monomethyl ether methyl ester (see section A III).

Mono-2:4-dinitrophenylhydrazone of the aldehyde $C_{13}H_{14}O_5$. An ethanol solution of the $C_{13}H_{14}O_5$ aldehyde on treatment with Brady's reagent gave an immediate precipitate which on recrystallization from methanol yielded deep-yellow needles, m.p. 214°. (Found: C, 53·42; H, 4·34; N, 14·0, 13·3; OCH_3, 14·25. $C_{17}H_{12}O_8N_4(OCH_3)_2$ requires C, 53·02; H, 4·21; N, 13·0; OCH_3, 14·4%.) The substance is insoluble in aqueous $NaHCO_3$, Na_2CO_3, cold NaOH or dilute ammonia, but dissolves slowly in NaOH on heating producing a brown solution. The substance, therefore, does not contain a free carboxyl group, although it may have a lactone group.

Oxidation of the aldehyde, $C_{13}H_{14}O_5$, *with alkaline iodine. Formation of a monobasic acid monolactone,* $C_{12}H_{14}O_6$. The aldehyde, $C_{13}H_{14}O_5$ (1·0 g.), was dissolved in methanol (5 ml.) by warming, cooled and treated with 0·1N-iodine (200 ml.) with vigorous shaking. 0·1N-NaOH (250 ml.) was added, and the whole was well shaken until all the oil disappeared. The liquid became colourless. After 30 min. N-H_2SO_4 (25 ml.) was added and the liberated I_2 was titrated with 0·5N-$Na_2S_2O_3$. The colourless mixture was made acid to Congo red and extracted four times with an equal volume of ether. The ethereal extract was shaken with aqueous $NaHCO_3$ (20 ml.), and the bicarbonate solution on acidification gave colourless crystals, 0·24 g., m.p. 147°, which on recrystallization from ether-light petroleum gave colourless feathery needles, m.p. 151–152°. (Found: C, 58·72, 58·60; H, 5·14, 5·20; OCH_3, 23·4. $C_{11}H_8O_4(OCH_3)_2$ requires C, 58·64; H, 5·30; OCH_3, 23·3%.) 0·2023 g. of the acid was titrated in the cold to phenolphthalein with 0·1N-NaOH requiring 7·52 ml. corresponding to an equivalent of 269. $C_{13}H_{14}O_6$ titrating as a monobasic acid requires 266. A further 20·00 ml. of 0·1N-NaOH was added and the solution was boiled under reflux, with exclusion of CO_2, for 2·5 hr. A back-titration used 12·48 ml. of 0·1N-H_2SO_4. Thus the additional acid groups unmasked were equivalent to 7·52 ml. 0·1N-alkali. A second acidic group has thus been revealed by alkaline hydrolysis; the substance behaves as a mono-acid monolactone.

In a second oxidation experiment, in which a proportionately much larger amount of methanol was used ($C_{13}H_{14}O_5$ aldehyde, 0·25 g.; methanol, 7 ml.; 0·1N-I_2, 60 ml.; 0·1N-NaOH, 75 ml.), the products extracted by ether, after acidification, were separated into acidic and neutral fractions by means of $NaHCO_3$. The *acidic fraction* yielded the acid $C_{13}H_{14}O_6$, m.p. 151–152°, referred to above. The *neutral fraction* yielded, after several recrystallizations from ether, large colourless prisms, m.p. 94–95°. (Found: C, 59·88; H, 5·92; OCH_3, 33·1, 32·9. $C_{11}H_7O_3(OCH_3)_3$ requires C, 59·98; H, 5·75; OCH_3, 33·2%.) This product thus appears to be the *methyl ester of the acid lactone* $C_{12}H_{14}O_6$, m.p. 151–152°. Its formation must be due to the use of methanol to dissolve the aldehyde prior to oxidation.

Oxidation of the monobasic acid monolactone, $C_{13}H_{14}O_6$, *with* $KMnO_4$. *Formation of a tribasic acid,* $C_{12}H_{12}O_7$. The monobasic acid monolactone $C_{13}H_{14}O_6$ (0·5 g.) was boiled for 1·5 hr. under reflux with N-NaOH (10 ml.) and water (50 ml.) to open the lactone ring. When cold the solution was treated with a cold solution of $KMnO_4$ (0·4 g., equivalent to 2 atoms O) in water (50 ml.). The mixture was left for 3 days at room temperature. The precipitated MnO_2 was removed by filtration. The pale-green filtrate was acidified to Congo red with H_2SO_4 and extracted with ether. On removal of the solvent a solid residue was obtained, 0·56 g., which proved to be a mixture of substances. It was fractionated by solution in water, neutralization with NaOH, and precipitation with aqueous neutral lead acetate (10 ml. of 0·5N). The white precipitate thus obtained was collected, washed with water, acidified with H_2SO_4 and extracted with ether. On removal of the ether the dry product (0·19 g.) obtained was recrystallized from ether-light petroleum, giving 0·10 g. of colourless slender prisms, m.p. 161–162°, raised to 162·5° by sublimation *in vacuo*. (Found: C, 55·80; H, 4·37; OCH_3, 23·0; neutralization equivalent in cold 96·8. $C_{11}H_6O_5(OCH_3)_2$ requires C, 55·71; H, 4·32; OCH_3, 22·1%; equivalent, titrating as a tribasic acid, 93·4.)

III. *Action of ozone on mycophenolic acid monomethyl ether methyl ester.* Mycophenolic acid monomethyl ether methyl ester (5·6 g.), m.p. 58° (Clutterbuck & Raistrick, 1933, p. 657), in chloroform (250 ml.) was ozonized for 10 hr.

Water fraction. This fraction contained very little material giving a precipitate with Brady's reagent. Its ether extract

was split as in sections AI and AII, by extraction with aqueous $NaHCO_3$, into (*a*) neutral fraction, 0·2 g. oil; (*b*) acid fraction, 0·15 g. oil. Neither fraction was examined further.

Chloroform fraction. The acid fraction, 0·12 g. of crystals, was not further examined. The neutral fraction, 5·4 g. of oil, was distilled *in vacuo* and gave the following fractions:

Fraction 1, b.p., 45–55°/1 mm.; 0·86 g.; mobile liquid.

Fraction 2, b.p., 190°/1 mm.; 0·96 g.; viscous oil, depositing crystals.

Fraction 3, b.p., 195°/<1 mm.; 0·96 g.; viscous oil, depositing crystals.

Fraction 4, undistilled residue. Some decomposition. Not further examined.

Fraction 1: 0·1 g. treated with Brady's reagent (50 ml.) gave crystalline orange needles, 0·16 g., m.p. 137°. It recrystallized readily from ethanol in bright-orange flattened needles, m.p. 137–138°, which did not depress the melting point of the mono-2:4-dinitrophenylhydrazone of synthetic methyl levulate, m.p. 139°. (Found: C, 46·56, 46·59; H, 4·65, 4·45; N, 18·3; OCH_3, 10·1. $C_{11}H_{11}O_5N_4(OCH_3)$ requires C, 46·44; H, 4·55; N, 18·05; OCH_3, 10·0%.)

Fractions 2 and 3 were combined and pressed on porous tile, giving 0·87 g. of colourless crystals, which after recrystallization from light petroleum, b.p. 40–60°, or water gave a strong Schiff reaction, and melted at 112° alone or in admixture with the aldehyde $C_{13}H_{14}O_5$ obtained by ozonization of mycophenolic acid monomethyl ether (see section AII). (Found: C, 62·15, 62·33; H, 5·75, 5·81; OCH_3, 24·7; neutralization equivalent in hot solution, 240. $C_{11}H_8O_3(OCH_3)_2$ requires C, 62·40; H, 5·64; OCH_3, 24·8%; neutralization equivalent, titrating as a monobasic acid, 250.) It also gave the same 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 214–215°. (Found: C, 53·10; H, 4·31; OCH_3, 14·2. $C_{17}H_{12}O_6N_4(OCH_3)_2$ requires C, 53·02; H, 4·21; OCH_3, 14·4%.)

B. *Acetylation of the aldehyde,* $C_{13}H_{14}O_5$. *Formation of an enol mono-acetate*

The aldehyde, m.p. 112° (0·10 g.), was heated with anhydrous sodium acetate (0·2 g.) and acetic anhydride (0·4 ml.) until the mixture began to boil and was then maintained at 140° for 0·5 hr. There was only a slight discoloration. The mixture was poured into water and stirred. A white flocculent precipitate (0·08 g., m.p. 130–133°) separated. Recrystallization from water and light petroleum, b.p. 80–100°, gave colourless needles, m.p. 140°. (Found: C, 61·30; H, 5·70; $CH_3.CO$, 17·0; $CH_3(C)$, 10·6. $C_{11}H_7O_5(CH_3CO)(CH_3)_2$ requires C, 61·65; H, 5·52; $CH_3.CO$, 14·7; $2CH_3(C)$, 10·3%.) The product gave no reaction with 2:4-dinitrophenylhydrazine (Brady's reagent) or with Schiff's reagent. It is thus clearly an enol mono-acetate of the $C_{13}H_{14}O_5$ aldehyde.

Ozonization of the enol acetate. Formation of an aldehyde, $C_{12}H_{12}O_5$. A solution of the enol acetate (0·2 g.) in chloroform (20 ml.) was ozonized for 45 min. After treatment with hot water the chloroform layer was separated, washed with a little aqueous $NaHCO_3$, dried with Na_2SO_4 and evaporated. The oil (0·12 g.) remaining partly crystallized *in vacuo*. The whole was crystallized from light petroleum, b.p. 80–100°, and gave 25 mg. of colourless needles, m.p. 118°, which were sublimed in a high vacuum giving a colourless crystalline sublimate m.p. 125–126°. (Found: C, 60·36; H, 4·92. $C_{12}H_{12}O_5$ requires C, 61·01; H, 5·12%.) The product gave a precipitate with Brady's reagent and a positive reaction with Schiff's reagent.

The residual oil (90 mg.), recovered from the light petroleum mother liquors, was treated with ethanol (2 ml.) and Brady's reagent (60 ml.). The crude red-orange 2:4-*dinitrophenylhydrazone* (115 mg.) was recrystallized from benzene, and gave 60 mg. of red-brown glistening needles containing one molecule of benzene of crystallization. On heating there was slight softening at 212–214°, with fairly sharp melting at 228–230° with slight effervescence. (Found, on air-dried crystals: C, 58·60; H, 4·51; N, 11·8; loss in high vacuum at 100–110°, 19·7. $C_{18}H_{16}O_5N_4.C_6H_6$ requires C, 58·30; H, 4·49; N, 11·3; loss 15·8%. Found, on crystals dried to constant weight: C, 51·76; H, 3·96; N, 13·3. $C_{18}H_{16}O_5N_4$, i.e. dinitrophenylhydrazone of $C_{12}H_{12}O_5$, requires C, 51·94; H, 3·88; N, 13·45%.)

C. *Colour reactions of mycophenolic and normycophenolic acids with 2:6-dichloroquinone chloroimide*

A small amount of each acid was dissolved in 2–3 ml. of sodium borate buffer, pH 9·2. To each solution was added two drops of a suspension of 2:6-dichloroquinone chloroimide in water. Mycophenolic acid gave a very faint dirty yellow colour, unchanged after 40 min. Normycophenolic acid rapidly gave a colour, blue with a tinge of green, inclining after 40 min. to purple. A control experiment using buffer solution and quinone alone gave a very faint pink unchanged after 40 min.

D. *Carbon-methyl group estimations*

(*a*) On the aldehyde $C_{13}H_{14}O_5$. Found: $CH_3(C)$, 4·75%. $C_{13}H_{14}O_5$ with one $CH_3(C)$ group requires 6·00%. This corresponds to 79% of the theoretical for one $CH_3(C)$ group.

(*b*) On mycophenolic acid. Found: $CH_3(C)$, 6·57%. $C_{17}H_{20}O_6$ with two $CH_3(C)$ groups requires 9·38%. This corresponds to 140% of the theoretical for one $CH_3(C)$ group and to 70% of the theoretical for two $CH_3(C)$ groups.

SUMMARY

1. Further degradative experiments have been carried out on mycophenolic acid, a metabolic product of species and strains in the *Penicillium brevicompactum* Dierckx series. These experiments consist mainly of a study of the products formed by the ozonolysis of mycophenolic acid and some of its methylated derivatives and breakdown products.

2. They lead to two possible structural formulae for mycophenolic acid, one being more probable than, and preferred to, the other. Mycophenolic acid may be described as 6-hydroxy-4-methoxy-3(or 5)-methyl-5(or 3)-(5′-carboxy-3′-methylpent-2′-enyl) phthalide.

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The Oxidation of Diiodotyrosine Derivatives

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It is now well established that when diiodotyrosine is incubated under mildly alkaline conditions, thyroxine is formed (von Mutzenbecher, 1939; Block, 1940; Johnson & Tewkesbury, 1942; Harington & Pitt-Rivers, 1945); under the most favourable conditions, however, the yields of thyroxine are small, and a large proportion of the diiodotyrosine is lost, giving rise to tarry by-products, inorganic iodide and ammonia. So far, no compound has been isolated after such an incubation which would indicate through what intermediate step thyroxine is synthesized; nor has the side chain, which is removed from one molecule of diiodotyrosine during the coupling of two molecules to give thyroxine, or a recognizable derivative thereof, been isolated, although Johnson & Tewkesbury (1942) claim to have detected pyruvic acid in the reaction mixture when thyroxine is formed under these conditions.

It seemed possible that further investigation of the products of alkaline oxidation of diiodotyrosine might reveal some compound which would give a clue to the mechanism of the reaction. It had been shown by Harington & Pitt-Rivers (1945) that the pH at which diiodotyrosine is incubated exerts a marked effect both on the amount of thyroxine formed and on the extent to which diiodotyrosine itself is oxidized. At a high pH (13 or 14) no thyroxine was obtained by these authors, but instead another crystalline iodine-containing compound was isolated, which was not characterized at the time. This compound has now been re-examined and has been identified as 3:5-diiodo-4-hydroxybenzaldehyde. Further examination of the products of incubation of diiodotyrosine at pH 10 has shown that at this pH the aldehyde is formed at the same time as thyroxine; at lower pH (7·8–8·8) none of the aldehyde is obtained, while the yields of thyroxine are very small.

The simultaneous formation of 3:5-diiodo-4-hydroxybenzaldehyde and thyroxine from diiodotyrosine led first to the hypothesis that the aldehyde might be an intermediate in thyroxine synthesis, and attempts were made to demonstrate this by incubating diiodotyrosine in the presence of the aldehyde, in the hope that increased yields of thyroxine might result. These attempts have proved abortive, and in all the experiments done the yield of thyroxine was unchanged and the aldehyde was recovered at the end of the reaction. Further, Dakin's (1909) observation that 3:5-diiodo-4-hydroxybenzaldehyde is unaffected by alkaline hydrogen peroxide, while the dichloro and dibromo analogues give more or less good yields of the corresponding quinols, has been confirmed. Only under the most drastic conditions may the diiodo aldehyde be oxidized, and the product of oxidation is 2:4:6-triiodophenol. These facts have, therefore, led to the abandonment of the hypothesis that the aldehyde is an intermediate in thyroxine synthesis, and it is now thought that its formation from diiodotyrosine is due to a different mechanism from that which leads to thyroxine.

The mode of formation of 3:5-diiodo-4-hydroxybenzaldehyde from diiodotyrosine is itself of some interest. It has been found that, when the aldehyde is obtained by incubating diiodotyrosine at pH 14, an amount of oxalic acid can be isolated as the calcium salt from the reaction mixture which is molecularly equivalent to the amount of aldehyde isolated; this leads to the suggestion that the

reaction entails, first, oxidation on the β carbon atom of the diiodotyrosine side chain. Further, when diiodotyrosine is incubated at pH 10, with the simultaneous formation of thyroxine and the aldehyde, oxalic acid is again obtained. In this instance the amount of oxalic acid appeared, in two experiments, to be molecularly equivalent to the sum of thyroxine and diiodo-*p*-hydroxybenzaldehyde isolated. This observation suggested that the aldehyde and thyroxine might be obtained by a mechanism whose initial steps were similar, but which, under the influence of pH or other factors (Pitt-Rivers, 1947), led to different end products. Even if we suppose that the apparent equivalence, mentioned above, of the oxalic acid formed with the sum of aldehyde and thyroxine produced at pH 10 is a genuine phenomenon, the observation is of little value in relation to the essential question of the form in which the side chain of one of the reacting molecules of diiodotyrosine is eliminated; oxalic acid is the common end product of oxidation of many 2- and 3-carbon aliphatic compounds, and in the present experiments its appearance could be explained equally well by the stepwise degradation of the side chain involving primary scission of the two terminal carbon atoms, or by secondary oxidation following elimination of the whole side chain as a 3-carbon fragment.

From what has already been said, it appears that the formation of 3:5-diiodo-4-hydroxybenzaldehyde from diiodotyrosine depends upon a certain degree of alkalinity, and in this connexion the formation of acid-insoluble iodine-containing compounds other than thyroxine during the preparation of artificial thyroproteins is called to mind.

During the preparation of such proteins by the method of Ludwig & von Mutzenbecher (1939), later developed by other workers (Reineke & Turner, 1942; Pitt-Rivers & Randall, 1945), the initial pH of the protein solution in bicarbonate buffer is about 8 and may rise to 9 during the iodination; it may also be remembered that Deanesly & Parkes (1945) showed that the method of assay as used for thyroid powder (determination of the acid-insoluble iodine content) cannot be applied to the artificial thyroproteins, since they contain large amounts of acid-insoluble iodine-containing products which are biologically inactive. The inactivity of 3:5-diiodo-4-hydroxybenzaldehyde as measured by the tadpole-metamorphosis test, together with the effect of alkaline conditions on aldehyde production from diiodotyrosine, suggested that this aldehyde might be isolated from the products of baryta hydrolysis of such a thyroprotein. This has proved to be so, and in one instance the amounts of aldehyde and thyroxine isolated were in the ratio of 10 : 1. No aldehyde has been isolated following baryta hydrolysis of desiccated thyroid powder.

The instability of diiodotyrosine itself is so great that it does not permit readily of its use in determining in what form the 3-carbon side chain is eliminated during thyroxine synthesis. In a further attempt to elucidate this point, a method of stabilizing the side chain has therefore been sought, and it was thought probable that acetylation of the amino group might achieve this result. Preliminary experiments on the alkaline incubation of *N*-acetyl-L-diiodotyrosine in solutions with a pH range of 7–8·5, followed by alkaline hydrolysis and butanol fractionation of the products, led to the isolation of thyroxine in small yields. During these incubations, however, it was noticed that a crystalline salt separated. This proved to be the sodium salt of *N*-acetyl-L-thyroxine, and from it the free acid could be obtained in yields of about 25%, calculated on the amount of unchanged starting material recoverable at the end of the experiments. *N*-Acetyl-L-diiodotyrosine has been titrated potentiometrically, and the pH optimum for acetyl thyroxine formation has been determined. Acid hydrolysis of acetyl thyroxine in a mixture of acetic acid and concentrated hydrochloric acid yielded L-thyroxine with $[\alpha]_D^{21°} - 5{\cdot}42°$ in 3% solution in a mixture of two parts ethanol and one part N-NaOH.

In the thyroid gland itself, thyroxine must, in part, be formed from diiodotyrosine molecules, which are joined through both their amino and carboxyl groups in peptide linkage; it appeared, therefore, to be of interest to discover whether a diiodotyrosine derivative, in which both these groups are blocked, would lead *in vitro* to the formation of a corresponding thyroxine derivative, and *N*-acetyl-DL-diiodotyrosylglutamic acid was prepared for this purpose. Incubation of the acetyl peptide at pH 7·2 in dilute sodium hydroxide solution led to no insoluble salt of a thyroxine derivative, but alkaline hydrolysis of the reaction mixture did yield a small amount of thyroxine. If the incubation of the acetyl peptide were carried out at pH 7·2 in baryta, however, a partly crystalline barium salt separated which, after decomposition with acid, yielded *N*-acetyl-DL-thyroxyl-L-glutamic acid. This thyroxine derivative has been obtained in yields up to 36% net, and conversion of the diiodotyrosine derivative to the thyroxine derivative is particularly good in this instance, gross yields being of the order of 20%. Acid hydrolysis of acetylthyroxylglutamic acid led to the isolation of thyroxine and glutamic acid.

The good yields of acetylthyroxine and the acetylthyroxine peptide obtained in these experiments, together with the relative freedom of the reaction mixtures from tarry products, have led to the hope that the diiodotyrosine side chain which is detached might be isolated, either as an acetamido compound or, after hydrolysis, as an amino-acid. After 2 or 3 weeks' incubation of acetyldiiodotyrosine in

baryta and removal of acetylthyroxine and of as much as possible of the starting material, the residue was hydrolyzed with sulphuric acid and freed from diiodotyrosine, iodide and sulphate. The residual solution contained an amino-acid (positive ninhydrin reaction) and chromatographic examination, kindly carried out by Dr R. L. M. Synge, revealed in two separate experiments the presence of small amounts of alanine. Further experiments are being carried out in an effort to isolate this amino-acid, and to discover whether this represents the diiodotyrosine side chain which is split off. Serine and glycine have been looked for, but Dr Synge has reported that there is no evidence of their presence.

EXPERIMENTAL

Isolation and identification of 3:5-*diiodo*-4-*hydroxybenzaldehyde from* L-*diiodotyrosine after oxidation at* pH 14 *with* H_2O_2 (*hot*)

L-Diiodotyrosine.$2H_2O$ (28·2 g.) was dissolved in a mixture of 12 ml. 10 N-NaOH and 100 ml. 2 N-NaOH, and heated on a boiling water bath; 20 vol. H_2O_2 (60 ml.) were added, and after the vigorous reaction had subsided the solution was cooled and extracted with an equal volume of *n*-butanol; these operations were repeated three times. The pooled butanol extracts were washed once with half their volume of 2 N-NaOH and concentrated to dryness. The crystalline residue was dissolved in water (200 ml.) and acidified at the boiling point with 14 N-H_2SO_4. A white crystalline precipitate immediately appeared which was collected, washed with water and dried *in vacuo* over P_2O_5. The yield was 1·11 g. After recrystallization from benzene, 0·71 g. of the product was obtained (prisms), and had m.p. 194–196° (decomp.) not depressed in admixture with authentic 3:5-diiodo-4-hydroxybenzaldehyde. (Found: C, 22·6; H, 1·2; I, 68·0. Calc. for $C_7H_4O_2I_2$: C, 22·5; H, 1·07; I, 67·9%.)

Reduction of the above compound. 0·42 g. was dissolved in 7 ml. N-NaOH and 20 ml. water, and hydrogenated in the presence of 0·25 g. palladized strontium carbonate. At the end of 2 hr. the reduction was complete and 40 ml. of hydrogen had been taken up. The reaction mixture was filtered, treated with 1·4 ml. 5 N-H_2SO_4 and concentrated to dryness. The residue was crystallized from the minimum amount of hot water. The aqueous mother liquor was again concentrated to dryness and crystallized from water; 90 mg. product were obtained with m.p. 111–112°, and were recrystallized from water in needles with m.p. 114–115°. The addition of *p*-hydroxybenzaldehyde did not depress the melting point.

The product formed a semicarbazone with m.p. 218–220° which, with *p*-hydroxybenzaldehyde semicarbazone, had m.p. 219–220°. (Found: N, 23·0. Calc. for $C_8H_9O_2N_3$: N, 23·5%.)

Isolation of 3:5-*diiodo*-4-*hydroxybenzaldehyde and oxalic acid after incubation of* L-*diiodotyrosine for* 14 *days at* pH 14

Diiodotyrosine.$2H_2O$ (46·9 g.) was dissolved in a mixture of 300 ml. N-NaOH and 20 ml. 10 N-NaOH, and incubated for 14 days, after which the solution was extracted once with an equal volume of butanol. After washing the butanol extract with about 50 ml. N-NaOH it was concentrated to dryness. It yielded 0·308 g. crystalline product with m.p. 184–190° (decomp.) with previous darkening. The product was then extracted repeatedly with hot 20% sodium metabisulphite solution, decomposition of which, with mineral acid, gave 0·235 g. of 3:5-diiodo-4-hydroxybenzaldehyde, m.p. 198–200° (decomp.); the mixed melting point with the authentic aldehyde was not depressed. The aqueous fraction was freed from butanol by a short distillation under diminished pressure, and the pH was brought up to 4·3 by addition of conc. HCl. The diiodotyrosine which separated was allowed to crystallize at 4° during 4–5 hr. and was then collected, washed with a little water and air-dried. Recovered: diiodotyrosine, 44·8 g.

The filtrate was brought to pH 5·5 with NaOH and treated with 2 ml. 5 M-$CaCl_2$ solution. After a short time calcium oxalate began to separate, and crystallization was completed by leaving at room temperature for 15 hr. The precipitate was collected on the centrifuge, washed with water and determined by titration with 0·1 N-$KMnO_4$. Oxalic acid found: 47 mg. The volume of the solution from which the calcium oxalate had separated was 850 ml. Calcium oxalate is soluble in water at 25° to the extent of 0·7 mg./100 ml., an a correction for this was made bringing the oxalic acid to 51·2 mg. (0·57 mmol.). The amount of diiodo-*p*-hydroxybenzaldehyde found (0·235 g.) was 0·63 mmol.

Formation of oxalic acid during incubation of diiodotyrosine at pH 10

(*a*) Diiodotyrosine.$2H_2O$ (46·9 g.) was incubated at pH 10 as described by Harington & Pitt-Rivers (1945). The butanol-soluble fraction yielded acid-insoluble material weighing 1·08 g. This was extracted three times with about 25 ml. ether, then twice with 20 ml. ethyl acetate and again with ether. The ether-insoluble portion, on dissolving in boiling 0·1 N-Na_2CO_3 and working up as usual gave 0·12 g. thyroxine.

The ether-ethyl acetate-soluble portion after removal of solvent weighed 0·82 g. Extraction with hot 20% sodium metabisulphite solution, until no further material would dissolve, gave in all 0·46 g. diiodo-*p*-hydroxybenzaldehyde, m.p. 196–198° (decomp.).

From the aqueous fraction, 41·68 g. diiodotyrosine were recovered. 5 M-$CaCl_2$ solution was added to the filtrate and the precipitated oxalate was collected, redissolved in acid, filtered from a small amount of brownish material, reprecipitated, collected by centrifuging and washed with water. The calcium oxalate was then dissolved in N-H_2SO_4 and the solution was divided into halves. A determination of oxalate by permanganate titration was made on the one half, while the other half was extracted many times with ether. The ether extract on evaporating to dryness gave crystals which on recrystallization from water had m.p. 100–101°, not depressed in admixture with oxalic acid dihydrate. The permanganate titration showed that there was present 0·190 g. calcium oxalate (1·46 mmol.). Thyroxine isolated was 0·12 g. (0·155 mmol.); diiodo-*p*-hydroxybenzaldehyde was 0·46 g. (1·23 mmol.).

(*b*) A repetition of the above experiment on 35·2 g. diiodotyrosine.$2H_2O$ with 7 days' incubation gave thyroxine 39 mg. (0·05 mmol.), diiodo-*p*-hydroxybenzaldehyde 0·28 g. (0·75 mmol.) and oxalic acid 0·78 g. (0·87 mmol.).

Incubation of diiodotyrosine at pH 7·8

This was done in exactly the manner described by Harington & Pitt-Rivers (1945). The butanol extract gave an acid-insoluble crude product weighing 69 mg. After extraction with ether, this fraction weighed 15 mg., from which 8 mg. pure thyroxine were obtained. The ether extract on being concentrated to dryness and treated with metabisulphite gave no aldehyde which could be weighed, although a trace of material (less than 1 mg.) was seen in the acidified metabisulphite solution which crystallized in the characteristic prisms of diiodo-*p*-hydroxybenzaldehyde. The recovered diiodotyrosine weighed 8·09 g., which gave a net yield of thyroxine of 0·74%.

Products obtained from the hot peroxide oxidation of diiodotyrosine

L-Diiodotyrosine (23·5 g.) was dissolved in 0·96 N-NaOH (99 ml.) and heated on a boiling water bath. Butanol (150 ml.) was added, and then 20 vol. H_2O_2 (150 ml.) was added in portions with continuous vigorous shaking. When there was no further effervescence the reaction mixture was cooled and the butanol layer separated. This was repeated twice, using 250 ml. butanol for the subsequent extractions. The pH was kept just alkaline to phenolphthalein throughout the reaction by small additions of N-NaOH; each butanol extract was washed with 100 ml. N-NaOH and the pooled washings were re-extracted with 250 ml. butanol.

The butanol-soluble fraction contained 3·18 g. of acid-insoluble material. This was extracted seventeen times by grinding with ether. The ether-insoluble portion gave 0·25 g. thyroxine after purification through the sodium salt.

The ether extract was concentrated to dryness. Repeated extraction with hot 20% sodium metabisulphite gave 0·29 g. of a crystalline compound, m.p. 198–199° (decomp.), mixed melting point with 3:5-diiodo-4-hydroxybenzaldehyde, 200–201° (decomp.). The residue after bisulphite extraction was steam distilled; the steam-volatile material weighed 0·144 g. and had m.p. 154–156° not depressed by mixing with 2:4:6-triiodophenol. (Found: I, 80·5 and 81% on duplicate analysis. Calc. for $C_6H_3OI_3$: I, 80·7%.)

The aqueous residue after butanol extraction was added to the butanol washings and acidified at the boiling point. A dark-brown tar separated which solidified on cooling; it was ground with water, collected and dried, and weighed 7·45 g. It contained no steam-volatile material.

The tar (5 g.) was dissolved in N-KOH (165 ml.) and reduced with hydrogen in the presence of palladized strontium carbonate (15 g.). At the end of the reduction the solution was filtered from catalyst, concentrated to a low volume and acidified with 5N-HCl; 0·75 g. of insoluble material was precipitated and was extracted several times with ether. The ether extract on evaporating to dryness gave a solid residue which, on crystallization from water, yielded 35 mg. of a crystalline compound with m.p. 208°. The melting point of this compound was not depressed by mixing with *p*-hydroxybenzoic acid with m.p. 211–213°.

Of the original unreduced tar, 0·4 g. was extracted with warm saturated sodium acetate solution. The extract was diluted with two parts of water and acidified at the boiling point with conc. HCl. Sheaves of needles separated on cooling and weighed 30 mg. They had m.p. about 220° (decomp.) with loss of iodine above 200°. Mixed melting point with 3:5-diiodo-4-hydroxybenzoic acid was not depressed.

Preparation of biologically active iodinated casein and isolation therefrom of thyroxine and diiodo-p-hydroxybenzaldehyde

Casein (Kahlbaum nach Hammarsten) (50 g.) was suspended in water (2 l.) and dissolved with the aid of 2 N-NaOH. $NaHCO_3$ (15 g.) was added and the pH was brought back to 8 by addition of 2N-HCl; 19 ml. of a 4·16 N-I_2 solution were added over a period of 2·5 hr. while the solution was mechanically stirred at 40°. The temperature was then raised to between 60 and 70° and maintained there for 5 hr., at the end of which the pH was 8·9 (British Drug Houses Ltd. capillator).

The iodinated casein was then precipitated by bringing the reaction mixture to pH 4 by addition of HCl. The separated protein was redissolved in the same volume of water by addition of alkali, reprecipitated with acetic acid, collected, washed with about 450 ml. N/700 acetic acid and air-dried. After some accidental loss the yield was 38 g.

Of this preparation 35 g. were boiled for 20 hr. under reflux with 500 ml. water and 200 g. recrystallized $Ba(OH)_2$. The hydrolysate was filtered hot, and the decomposed barium salt was combined with the acid-insoluble material in the filtrate. This product weighed 0·82 g.; it was extracted twice with ether, once with ethyl acetate and then three times again with ether. The residue weighed 0·78 g., and yielded in all 108 mg. pure thyroxine, from which the thyroxine content of the protein was 0·31%. The pooled ether and ethyl acetate extracts were combined and evaporated to dryness, and the crystalline residue was purified by extraction with hot 10% sodium metabisulphite. The crystalline compound obtained by decomposition of this extract weighed 15 mg. and had m.p. 197–198° (decomp.). The mixed melting point with authentic 3:5-diiodo-4-hydroxybenzaldehyde was not depressed.

Isolation of 3:5-diiodo-4-hydroxybenzaldehyde from iodinated proteins with low biological activity

The method was as follows: 50–100 g. protein were boiled with 20 parts 2 N-NaOH for 18 hr., and then cooled, strained through glass wool if necessary, and extracted once with an equal volume of butanol. The butanol fraction was worked up as described in the previous hydrolysis.

Iodinated casein (*PB*11) *DT*/5/829 contained 8·9% total I and 3·3% acid-insoluble I (see Deanesly & Parkes, 1945); 60 g. yielded 23 mg. of pure thyroxine (giving 0·038% thyroxine) and 45 mg. (0·075%) pure diiodo-*p*-hydroxybenzaldehyde.

Iodinated casein (*PB*6) *DT*/5/823 contained 7·4% total I and 2·3% acid-insoluble I (see Deanesly & Parkes, 1945). Hydrolysis of 50 g. of this preparation for 7 hr. with 4N-NaOH, followed by butanol extraction, yielded no thyroxine and only 5–6 mg. pure aldehyde.

*Hydrolysis of a mixture of iodinated caseins PB*6 *and PB*11 *with fractionation of acid-insoluble* I

*PB*6 (50 g.) + *PB*11 (15 g.) were boiled under reflux for 16·5 hr. with 1 l. 2N-NaOH. After cooling, the hydrolysate was extracted with an equal volume of butanol. This gave

an acid-insoluble precipitate of 1·05 g. containing 27·8% I, and after purification through the usual processes gave thyroxine (3·0 mg.) and 3:5-diiodo-4-hydroxybenzaldehyde (30 mg.). The aqueous fraction contained 6·77 g. acid-insoluble material containing 5·51% I, from which no crystalline compound could be obtained.

Under these conditions of hydrolysis, it therefore appears that: (i) of the acid-insoluble iodine found (665 mg.) 56% is not soluble in butanol, and (ii) that of the butanol-soluble acid-insoluble iodine, less than 1% could be accounted for as thyroxine, while 7% was accounted for as diiodo-*p*-hydroxybenzaldehyde; 92% of this butanol-soluble iodine was not characterized.

Hydrolysis of desiccated thyroid powder B 8

Defatted thyroid powder (200 g.) was boiled under reflux with 2 l. 2N-NaOH for 17 hr., and fractionated into butanol as described above. The acid-insoluble residue from the butanol fraction weighed 5·6 g. This fraction was composed largely of fat and was largely soluble in ether. The ether-soluble material was boiled three times with 20% sodium metabisulphite solution, and each extract was cooled before filtering (since the bisulphite compound of 3:5-diiodo-4-hydroxybenzaldehyde is soluble in cold water, this procedure did not risk the loss of aldehyde). Decomposition of the bisulphite solution with mineral acid led to no insoluble material, so that no aldehyde appeared to be present. Since it was possible that the aldehyde might not be extractable from the fat with any ease, about 1 mg. authentic aldehyde was added to it: one bisulphite extract easily removed it.

Experiments on the incubation products of N-*acetyl-*L-*diiodotyrosine*

N-*Acetyl-*L-*diiodotyrosine.* This compound has already been prepared by Myers (1932), but the method is not suitable for large amounts. Moreover, the compound is unstable and tends to become coloured during recrystallization. The following method gives a product which may be used for incubation experiments without further crystallization. *ON*-Diacetyl-L-diiodotyrosine (Myers, 1932) (25·85 g.) was dissolved in 150 ml. cold N-NaOH. After 10 min. at room temperature, the solution was filtered, diluted to 600 ml. with water, warmed to 75° and acidified by addition of 170 ml. N-HCl with stirring; the product began to crystallize immediately and the solution was then cooled rapidly to 0°. After 2 hr. in the ice chest the crystals were collected, washed with water and dried *in vacuo* over P_2O_5; the yield was 22·5 g., m.p. 125°, $[\alpha]_D^{18°}$ +49·7° in 2% solution in 2N-NaOH. (Found: C, 27·3; H, 2·6; I, 52·9. Calc. for $C_{11}H_{11}O_4NI_2.\frac{1}{2}H_2O$: C, 27·3; H, 2·5; I, 52·5%.)

Apparent dissociation constant of the diiodophenolic group of N-*acetyl-*L-*diiodotyrosine.* The monosodium salt of *N*-acetyl-L-diiodotyrosine was titrated potentiometrically in 0·05M-solution at 23 and 37°. At 23° the apparent pK was 6·95; at 37° it was about 6·75, but at the higher temperature the compound was not stable, and the pK values calculated are not as constant as those obtained from the curve at 23°. This result is in fairly close agreement with the values calculated by Winnek & Schmidt (1935) for the apparent dissociation constant of the diiodophenolic group of diiodotyrosine at different temperatures. These authors found that the apparent pK calculated from solubility determinations was 6·53 at 0°, 6·48 at 25° and 6·45 at 40°.

Formation of thyroxine during aerobic incubation of N-*acetyl-*L-*diiodotyrosine.* *N*-Acetyl-L-diiodotyrosine (9·5 g.) was suspended in 100 ml. water, and dissolved by adding 37 ml. N-NaOH; the initial pH was 7·6. After 14 days at 37° the reaction mixture was made 4N with respect to NaOH and boiled under reflux for 1·5 hr. The cooled solution was diluted with an equal volume of water and extracted twice with an equal volume of butanol. Purification of the butanol-soluble fraction in the usual manner (Harington & Pitt-Rivers, 1945) yielded 144 mg. pure thyroxine; 5·25 g. diiodotyrosine was recovered from the aqueous fraction. The net yield of thyroxine was therefore 4·7%.

Effect of pH *on thyroxine formation.* *N*-Acetyl-L-diiodotyrosine was dissolved in water and different amounts of alkali, the initial pH of each solution being calculated from the titration curve. After 14 days at 37°, the thyroxine formed and residual diiodotyrosine were separated as described above. The results are given in Table 1.

Table 1. *Formation of thyroxine from* N-*acetyl-*L-*diiodotyrosine incubated at different* pH

Acetyl-diiodo-tyrosine incubated (g.)	pH	Thyroxine isolated (mg.)	Diiodo-tyrosine recovered (g.)	Net yield of thyroxine (%)
7·13	7·15	70	2·67	2·0
6·5	7·25	122	2·73	4·3
9·5	7·45	162	5·58	5·9
9·5	7·70	144	5·25	4·7
9·5	8·0	84	6·96	4·2
9·5	8·35	74	6·72	3·4

Isolation of N-*acetyl-*L-*thyroxine from the aerobic incubation products of* N-*acetyl-*L-*diiodotyrosine.* *N*-Acetyl-L-diiodotyrosine (9·5 g.) was dissolved in 100 ml. 0·05M-borate buffer at pH 7·6 and 17·8 ml. 2N-NaOH; the initial pH was 7·6. After 2 days at 37° a crystalline sodium salt began to separate, and this slowly increased in amount. After 12 days' incubation, the sodium salt was separated on the centrifuge and washed with a little water. Decomposition of this salt with dilute HCl led to the isolation of a crystalline compound weighing 290 mg. Recrystallization by the method of Ashley & Harington (1929) gave a product which crystallized in long thin prisms and which had m.p. 232–234° (decomp.); $[\alpha]_D^{25°}$ +25·7 in 4% solution in a mixture of equal volumes of ethanol and N-NaOH. (Found: I, 62·0. Calc. for *N*-acetylthyroxine, $C_{17}H_{13}O_5NI_4$: I, 62·0%.) From the mother liquor 8·25 g. *N*-acetyl-L-diiodotyrosine was recovered; the net yield of the acetylthyroxine was therefore 27%. Incubations of *N*-acetyl-L-diiodotyrosine in baryta solution at pH 7·6 led to the separation of the barium salt of *N*-acetyl-L-thyroxine in net yields of 15–20% calculated on the recovered starting material.

L-*Thyroxine from* N-*acetyl-*L-*thyroxine*

N-Acetyl-L-thyroxine (1·64 g.) was boiled under reflux for 1 hr. with 25 ml. acetic acid and 15 ml. conc. HCl. The solution was then concentrated to dryness, and the residue was dissolved in boiling 0·1N-Na_2CO_3 from which the sodium salt of thyroxine separated. After recrystallization, the thyroxine weighed 1·25 g. and had m.p. 232° (decomp.);

$[\alpha]_D^{21°}$ −5·40° in 3·335% solution in two parts ethanol and one part N-NaOH. Since this rotation was considerably higher than that found for L-thyroxine by Harington & Pitt-Rivers (1945), it was thought that the L-thyroxine obtained above might be contaminated by a small amount of *N*-acetyl-L-thyroxine. A second hydrolysis with the same acid mixture was done for 2 hr. The L-thyroxine obtained had $[\alpha]_D^{21°}$ −5·42° in 3·28% solution in the same solvent. The specific rotation of the L-thyroxine was then determined in 3·25% solution in a mixture of 24 g. 0·5 N-NaOH and 56 g. ethanol, and was found to be $[\alpha]_D^{17·5°}$ −4·62°. This is in good agreement with the value obtained by Foster, Palmer & Leland (1936) for L-thyroxine from the thyroid gland; these authors found $[\alpha]_D$ −4·4° in the same solvent.

Attempted identification of the side chain of diiodotyrosine which is eliminated during thyroxine synthesis

N-Acetyl-L-diiodotyrosine (96 g.) was dissolved in 1·12 l. dilute baryta solution containing 1·78 equiv. $Ba(OH)_2$/mol. acetyldiiodotyrosine. The initial pH was 7·6 as measured by cresol red (British Drug Houses Ltd. capillator). After 3 weeks at 35° the barium salt of acetylthyroxine, which had separated, was collected on the filter, and after decomposition with acid 6·23 g. acetylthyroxine were obtained. The mother liquor was just acidified with dilute H_2SO_4. Much unchanged acetyldiiodotyrosine was precipitated together with $BaSO_4$, from which it was separated by solution in dilute alkali and reprecipitation with acid. The recovered acetyldiiodotyrosine weighed 63·0 g., whence the net yield of acetylthyroxine was nearly 22%. The filtrate was extracted with ether several times to remove some acetyldiiodotyrosine which has a significant water solubility. It was concentrated somewhat and made 2N with respect to H_2SO_4 and boiled under reflux for 1 hr. to hydrolyze any acetyl amino-acid present. The solution was then freed from H_2SO_4 with $Ba(OH)_2$ and concentrated to about half its volume. Addition of Ag_2SO_4 in excess precipitated the inorganic iodide liberated by decomposition of acetyldiiodotyrosine during the incubation. Sulphate ions were again removed with $Ba(OH)_2$, and the filtrate was concentrated to 200 ml. At this point, the HNO_2 reaction for *o*-diiodophenolic groups was positive, as was the ninhydrin reaction. It was therefore suspected that diiodotyrosine was present; so the solution was treated with excess lead acetate and allowed to stand for 12 hr. The bulky precipitate was then separated on the filter and the filtrate freed from Pb with H_2S. After concentration to a small volume, crystals separated which had m.p. 215–216° (decomp.). These crystals gave positive ninhydrin, Millon and HNO_2 reactions. The mixed melting point with authentic L-monoiodotyrosine was not depressed. The presence of this amino-acid is attributed to incomplete iodination of the tyrosine from which the *N*-acetyl-L-diiodotyrosine was prepared. Since monoiodotyrosine is a fairly soluble amino-acid, it was thought that its removal would best be effected by its hydrogenation to tyrosine, which was accordingly done using palladized strontium carbonate as catalyst. After the reduction the solution was acidified with H_2SO_4 and freed from iodide with Ag_2SO_4; it was then freed from sulphate ions, and concentrated to about 15 ml. After several days in the ice chest 0·52 g. of tyrosine had separated. This was removed, and the mother liquor (16 ml.) was examined by Dr R. L. M. Synge by paper chromatography. The predominant amino-acid was alanine; a little tyrosine was also present. Attempts to crystallize the alanine by concentrating the solution to a low volume and distilling in ethanol were unsuccessful.

In a second experiment, 26·85 g. *N*-acetyl-L-diiodotyrosine were incubated at 35° in 415 ml. baryta solution at pH 7·6 for 4 weeks. At the end of this time 1·69 g. acetylthyroxine were obtained, and after removal of unchanged starting material (15·6 g.) the filtrate was treated as in the first experiment. No monoiodotyrosine was detected. When the volume of the mother liquor had been concentrated to 25 ml., this was again examined by Dr Synge who found as before that alanine was the predominant amino-acid present. Attempts to isolate alanine as the azobenzenesulphonic acid salt also failed.

Experiments on the incubation products of N-acetyl-DL-diiodotyrosyl-L-glutamic acid

N-Acetyl-DL-diiodotyrosyl-L-glutamic acid. 2-Methy-l4(3′:5′-diiodo-4′-acetoxybenzyl) oxazolone (Gell, Harington & Pitt-Rivers, 1946) (15·5 g.) was dissolved in 120 ml. acetone and mixed with a solution of glutamic acid (7·35 g.) in 100 ml. N-NaOH. After 12 hr. at room temperature 31 ml. 2N-NaOH were added to the solution to hydrolyze the *O*-acetyl group. After 1 hr. acetone was removed under diminished pressure, the solution was diluted with water and acidified with 3N-HCl; the product, which crystallized in small prisms, was recrystallized by dissolving in warm methanol and diluting with water to turbidity; the yield was 14·2 g. (76% of the theoretical). The compound had m.p. 212–214° (decomp.). (Found: C, 30·8; H, 3·47; I, 40·9. $C_{16}H_{18}O_7N_2I_2.H_2O$ requires C, 30·9; H, 3·22; I, 40·8%.) On attempting to dehydrate this compound by drying *in vacuo* at 100°, about 5% of its weight was lost, and there was some decomposition.

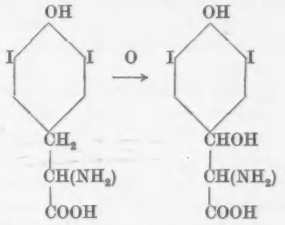
The isolation of N-acetyl-DL-thyroxyl-L-glutamic acid from the incubation products of the above peptide. Some preliminary incubations were done on *N*-acetyldiiodotyrosylglutamic acid in dilute NaOH solution at pH 7·2 and hydrolysis of the reaction mixture yielded some thyroxine; however, no insoluble sodium salt of the thyroxine derivative separated during the incubations. *N*-acetyl-DL-diiodotyrosyl-L-glutamic acid (11·8 g.) was dissolved in 155 ml. 0·341 N-$Ba(OH)_2$; the pH was 7·4 as measured with indicators. After a few hours at 37° a partly crystalline barium salt had started to separate and this greatly increased in the course of time. After 4 weeks, the barium salt was collected by filtration, washed with water and decomposed with dilute HCl. The product (2·3 g.) was crystallized by dissolving in cold methanol and diluting with water to turbidity, and separated in minute prisms which had m.p. 190–200° (decomp.). (Found: C, 27·8; H, 2·77; I, 52·5. *N-acetylthyroxylglutamic acid*, $C_{22}H_{20}O_8N_2I_4.H_2O$ requires C, 27·3; H, 2·3; I, 52·6%.) Attempts to dehydrate this compound at 100° led to some decomposition and loss of iodine. This thyroxine derivative does not form an insoluble sodium salt. Of *N*-acetyl-DL-diiodotyrosyl-L-glutamic acid 3·44 g. were recovered from the mother liquor, whence the net yield of the thyroxine derivative was 36%.

Acid hydrolysis of N-acetyl-DL-thyroxylglutamic acid. The peptide (1·93 g.) was boiled under reflux for 1·5 hr. with acetic acid (25 ml.) and conc. HCl (15 ml.). The solution was concentrated to dryness and the residue was extracted with

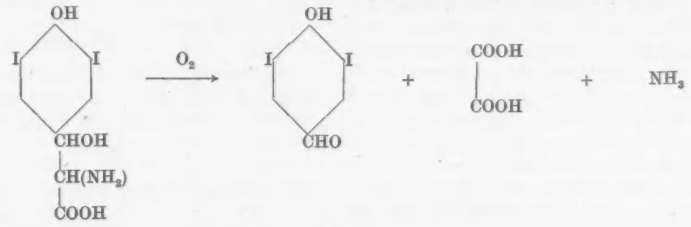
dilute HCl. The insoluble thyroxine was collected and purified in the usual manner. After crystallization the yield of thyroxine was 0·77 g. and it had m.p. 235° (decomp.). The filtrate was concentrated to a low volume and brought to pH 3·2 with NH_3. After a short time, triangular prisms began to separate. After 24 hr. in the ice chest these were collected, and the mother liquor was concentrated when more of the same crystals separated. The total yield of this compound was 0·163 g. After crystallization from water the compound had m.p. 204° (decomp.). The mixed melting point with authentic L-glutamic acid was not depressed. (Found: C, 40·9; H, 6·0; N, 9·7. Calc. for $C_5H_9O_4N$: C, 40·85; H, 6·1; N, 9·5%.)

DISCUSSION

The isolation of the side chain of diiodotyrosine as a 2-carbon residue (oxalic acid), after the formation of 3:5-diiodo-4-hydroxybenzaldehyde during oxidation at high pH values, suggests immediately the conclusion that the first step in the degradation of the amino-acid side chain under these conditions entails oxidation on the β carbon atom, with the formation of 3:5-diiodo-4-hydroxyphenylserine. By analogy with the finding of Bettzieche (1925), that phenylserine is broken down in alkaline solution to give benzaldehyde and glycine, the above hydroxy acid would, under the same conditions, give 3:5-diiodo-4-hydroxybenzaldehyde and glycine.



Now it is a little difficult to believe that the glycine molecule thus produced would, under the mild oxidative conditions prevailing during aerobic incubation at 38°, be oxidized quantitatively to oxalic acid (cf. Dakin, 1905–6, on the formation of glyoxylic acid and oxalic acid by the action of hydrogen peroxide on glycine). It is, therefore, suggested that the 3:5-diiodo-4-hydroxyphenylserine undergoes further oxidation on the α or β carbon atom, and subsequent fission to the aldehyde, oxalic acid and ammonia:



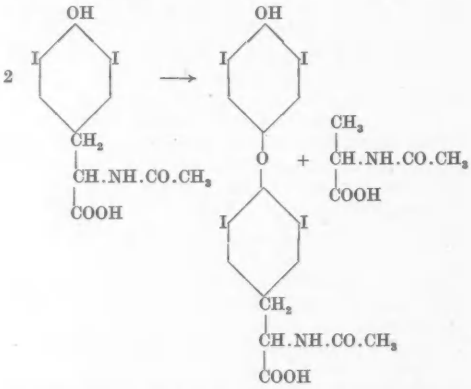
With regard to the formation of 3:5-diiodo-4-hydroxybenzaldehyde during the preparation of artificial thyroproteins, this may be simply a function of the pH of the reaction. The absence of this aldehyde from the thyroid is exceedingly probable, but has not yet been demonstrated conclusively, since the sample of B.P. thyroid used for an attempted isolation had been defatted, and any aldehyde present would be removed by any fat solvent. The experiment with thyroid powder was done in order to find out whether the aldehyde obtained from the iodinated casein might be an artifact of alkaline hydrolysis from diiodotyrosine present in the protein, and the negative result with thyroid powder showed that this was not so. For other reasons, however, it is unlikely that the aldehyde is formed in the thyroid gland *in vivo*: first, because the pH of the tissues is below that at which aldehyde formation occurs during the incubation of diiodotyrosine; and secondly, because Harington & Randall (1929) were able to account for all the acid-insoluble iodine in the thyroid as being thyroxine iodine.

The formation of 2:4:6-triiodophenol from diiodotyrosine after hot peroxide oxidation calls for some comment. It has been found, during a repetition of Dakin's (1909) experiments on the formation of quinols from hydroxybenzaldehydes, that 3:5-diiodo-4-hydroxybenzaldehyde, while it gives no quinol with hydrogen peroxide, can, under vigorous conditions of oxidation, lose the formyl group with the production of 2:4:6-triiodophenol besides much free iodine and iodoform. No triiodophenol is formed from diiodotyrosine under mild oxidative conditions, which indicates that the triiodophenol obtained from diiodotyrosine during hot peroxide oxidation is a secondary breakdown product formed from the aldehyde; the formation of this compound is, therefore, not considered to have any importance in elucidating the mechanism of thyroxine synthesis.

The original suggestion by Harington & Barger (1927) that thyroxine was formed by the coupling of two molecules of diiodotyrosine was amplified first by Johnson & Tewkesbury (1942). These authors postulated that during incubation of diiodotyrosine under von Mutzenbecher's (1939) conditions hypoiodite is formed. This oxidizes one molecule of diiodotyrosine to a quinonoid intermediate possessing a

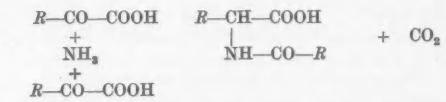
free radical, which unites with a further molecule of diiodotyrosine with univalent oxygen, the side chain being split off as pyruvic acid and ammonia. Later Harington (1944) developed a theoretical mechanism for thyroxine synthesis based on electronic considerations, and leading to the same type of intermediate as that suggested by Johnson & Tewkesbury (1942).

The chromatographic identification of small amounts of alanine among the reaction products obtained by acid hydrolysis of the mother liquors, after acetylthyroxine has been formed from acetyldiiodotyrosine, may lead to a different mechanism. If we suppose that this alanine is derived from the side chain which has been eliminated from one molecule of acetyldiiodotyrosine during the coupling, then the overall reaction may be graphically represented as a dismutation.



No oxidative process is required for this representation, although it is known (Harington & Pitt-Rivers, 1945) that thyroxine is not formed from diiodotyrosine under anaerobic conditions, and iodine is, in fact, liberated from acetyldiiodotyrosine during the reaction. This can be demonstrated by the presence of inorganic iodide in the reaction mixture, even in experiments where the net yields of acetylthyroxine are high and the recovered acetyldiiodotyrosine is free from tarry products (i.e. incubation in borate buffer).

The presence of alanine and the absence of serine (and glycine) during thyroxine synthesis recall the metabolism of tryptophan by *Escherichia coli*, elucidated by Dawes, Dawson & Happold (1947). These authors have identified the tryptophan side chain chromatographically as alanine when indole is formed by *Esch. coli* in the presence of the retarding agent mepacrine, and no serine has been found. It is interesting to speculate whether the two reactions, resulting in thyroxine on the one hand, and indole on the other, may not follow the same metabolic pathway, although the causative factor is different in the two cases, the former being chemical, the latter enzymic. Acetylalanine might, on the other hand, be obtained as a by-product of an entirely different reaction. If we assume that Johnson & Tewkesbury's (1942) mechanism of thyroxine synthesis is correct, the side chain would be eliminated from acetyldiiodotyrosine as pyruvic acid and acetamide. Now Knoop's theory (Knoop, 1910; Knoop & Kertess, 1911) to explain the formation of amino-acids from keto acids *in vivo* postulated the intermediate formation of an acylated amino-acid:



This theory was based on Knoop's own findings from *in vivo* experiments and was supported by the work of Erlenmeyer & Kunlin (1899, 1902) and de Jong (1900), who studied the *in vitro* reaction with many keto acids; in particular, when pyruvic acid and ammonium carbonate reacted together in aqueous solution, acetylalanine was formed in good yield. The theory was much later re-established by the work of du Vigneaud & Irish (1938) in experiments on the transformation in dogs of one amino-acid isomer into its enantiomorph through the intermediate keto acid.

If this be the source of acetylalanine in the reaction at present being studied, it must be assumed that the acetamide liberated during deamination of the side chain is hydrolyzed to acetic acid and ammonia, since Herbst (1939) has shown that the product of condensation of pyruvic acid and acetamide itself is of an entirely different nature. One experimental result, however, can decide upon the genesis of the alanine which has been detected: Acetylalanine obtained *in vitro* from pyruvic acid and ammonia would be optically inactive, whereas alanine split off as acetylalanine from *N*-acetyl-L-diiodotyrosine would be optically active, as is the *N*-acetylthyroxine formed. Efforts are now being made to isolate either acetylalanine or alanine itself in sufficient quantity to determine this point. Until this is done, the mechanism of the formation of alanine during thyroxine synthesis cannot profitably be discussed.

SUMMARY

1. 3:5-Diiodo-4-hydroxybenzaldehyde and oxalic acid have been isolated in equimolecular amounts after aerobic incubation of diiodotyrosine at pH 14. These findings are discussed.

2. The above aldehyde and thyroxine are formed together during aerobic incubation of diiodotyrosine at pH 10.

3. 2:4:6-Triiodophenol, the aldehyde and thyroxine are all formed during the hot peroxide oxidation of diiodotyrosine.

4. 3:5-Diiodo-4-hydroxybenzaldehyde is formed during the iodination of proteins to give products with thyroid-like activity; the aldehyde itself has no biological activity as measured by the tadpole metamorphosis test.

5. *N*-Acetyl-L-thyroxine is formed in substantial yield during aerobic incubation of *N*-acetyl-L-diiodotyrosine. Acid hydrolysis of the thyroxine derivative yields L-thyroxine.

6. *N*-Acetyl-DL-diiodotyrosylglutamic acid has been prepared. Aerobic incubation of this peptide leads to the formation of good yields of *N*-acetyl-DL-thyroxylglutamic acid which, on acid hydrolysis, yields thyroxine and glutamic acid.

7. Alanine has been chromatographically identified among the reaction products obtained during thyroxine synthesis from diiodotyrosine. This finding is briefly discussed.

I wish to express my indebtedness to Dr C. R. Harington for his interest in this work. I have to thank Dr R. L. M. Synge for doing the chromatographic identifications of alanine; Dr W. J. Elford for his help in doing the potentiometric titration of *N*-acetyl-L-diiodotyrosine, and Dr A. S. Parkes for testing the biological activity of 3:5-diiodo-4-hydroxybenzaldehyde. I have also to thank Imperial Chemical Industries (Explosives) Ltd. for gifts of iodinated proteins.

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The Steroids of Pregnant Mares' Urine

1. A METHOD FOR THE EXTRACTION OF STEROID SULPHATES AND THE ISOLATION OF *allo*PREGN-16-EN-3(β)-OL-20-ONE SULPHATE*

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In a previous paper (Schachter & Marrian, 1938) dealing with the isolation of oestrone sulphate from the urine of pregnant mares, the isolation from the same source of what appeared to be the sulphate of a non-phenolic steroid was briefly noted. Fuller details were not reported at that time since concordant analyses upon the new sulphate and the parent steroid could not be obtained, and since there was some evidence to suggest that the material isolated might be a mixture of closely related sulphates varying in composition from batch to batch. More recently, it has been possible to commence a reinvestigation of the conjugated steroids in pregnant mares' urine, and in the present paper the methods used for the extraction and concentration of a steroid-sulphate fraction and the isolation and identification of one pure component of this fraction are described.

* For preliminary accounts of this work see *Biochem. J.* 1945, **39**, xlv; 1946, **40**, lv; 1948, **42**, i.

RESULTS

The method used for the extraction and concentration of the steroid-sulphate fraction was based on that of Schachter & Marrian (1938). It consisted of the following stages: (i) Extraction of the urine with *n*-butanol. (ii) Removal of much acidic matter from the material extracted with butanol, giving the 'crude conjugate fraction'. (iii) Treatment of an aqueous solution of the crude conjugate fraction with an equal volume of saturated potassium acetate, giving 'potassium acetate-soluble' and 'potassium acetate-insoluble' fractions. This stage served the double purpose of ensuring that K^+ was the only cation present in the insoluble fraction and of partially removing in the soluble fraction certain simpler organic sulphates such as that of *p*-cresol. (iv) Separation of the 'potassium acetate-insoluble' fraction into fractions readily and sparingly soluble in water, so-called 'water-soluble' and 'water-insoluble' fractions. (v) Repeated extraction of the 'water-insoluble' fraction with boiling acetone containing water (2% (v/v); '98% acetone'). On concentration and cooling to 0° these extracts yielded white solid material, '98% acetone solids, fraction *C*', which in some cases was crystalline. (In one batch of urine solid material (fraction *A*) separated from the 98% acetone extracts before concentration on cooling to 0°. In another batch, although no solid was obtained on cooling the unconcentrated extracts, crystalline material (fraction *B*) separated immediately when these extracts were heated in order to concentrate them. Fractions *A* and *B* will be dealt with in subsequent papers.)

The weights of certain fractions at the different stages of concentration for a number of batches of urine are summarized in Table 1.

On acid hydrolysis of the potassium or *p*-toluidine salt an ether-soluble substance (*Z*), the analysis of which agreed with the formula $C_{21}H_{32}O_2$, was obtained. *Z* yielded a monoacetate on treatment with acetic anhydride and pyridine, and gave a precipitate with digitonin in 90% (v/v) ethanol. It thus appeared to be a 3(β)-hydroxysteroid. No colour was given with tetranitromethane.

In the Zimmermann test *Z* gave a purple colour, although the development of the colour was slower than with neutral 17-ketosteroids, indicating the presence of a ketone group at some position other than C_{17}. *Z* did not, however, form a semicarbazone. The failure of *Z* to form a semicarbazone and the lack of colour with tetranitromethane were not inconsistent with the presence of an $\alpha\beta$-unsaturated ketone group (cf. Ruzicka, Huyser, Pfeiffer & Seidel (1929) on tetranitromethane reactions, and Allen & Blatt (1943) on semicarbazide reactions), and subsequently *Z* acetate was found to show the 240 and 320 mμ. absorption bands characteristic of $\alpha\beta$-unsaturated ketones.

The above evidence suggested that *Z* might be a pregnen- or *allo*pregnen-3(β)-ol-one having the double bond conjugated with the ketone group; its melting point and that of its acetate indicated that it might be *allo*pregn-16-en-3(β)-ol-20-one, which was obtained by Marker & Turner (1940) from dihydropseudotigogenin diacetate.

Samples of this *allo*pregnenolone acetate and of the 16:17-epoxy compound obtained from it by treatment with perbenzoic acid (Plattner, Ruzicka, Heusser & Angliker, 1947) were kindly supplied by Prof. Plattner of Zürich, and a comparison of these samples with *Z* acetate and its epoxy compound, respectively, showed that *Z* was indeed *allo*pregn-16-en-3(β)-ol-20-one. This appears to be the first reported isolation of a Δ^{16}-20-ketosteroid from urine.

Table 1. *Weights of conjugate fractions in standard extraction method*

(All batches of urine from mares in 9th-11th months of pregnancy. Results expressed as mg./l. urine.)

	No. of results	Mean	S.E. of mean	Range
Crude conjugate fraction	11	440	50	210–810
Potassium acetate-soluble fraction	8	40	8	20– 80
Potassium acetate-insoluble fraction	10	280	30	140–450
Water-soluble fraction	2	175	—	160–190
Water-insoluble fraction	10	120	20	50–210

Fraction *C* from certain batches of urine was found to consist largely of a single substance (*potassium Z sulphate*), which was obtained pure either directly by crystallization from water and from ethanol or via its *p*-toluidine salt (cf. Barton & Young, 1943). Analyses indicated the formulae

$$[C_{21}H_{31}OSO_4]^- K^+ . H_2O$$

and $$[C_{21}H_{31}OSO_4]^- [CH_3 . C_6H_4 . NH_3]^+$$

for the potassium and *p*-toluidine salts, respectively.

The batches of urine from which *Z* sulphate was readily isolated were collected 3 weeks or less before foaling, the yield of crude potassium *Z* sulphate being *c.* 25 mg./l. Other batches collected 4–16 weeks before foaling gave smaller quantities of cruder material, from which it was more difficult to obtain *Z* sulphate. This suggests that the amount of *Z* sulphate excreted, and the ratio of *Z* sulphate to related sulphates, both increase in the last few weeks before foaling.

EXPERIMENTAL

Melting points. These are corrected. Speed of heating at melting point, 3–4°/min.

Micro-analyses. We are indebted to Mrs G. F. Marrian for all micro-analyses, except those of compound *Z* which are by Drs Weiler and Strauss (Oxford).

Specific rotations. These are for the sodium *D* line; a 0·5 dm. micro-tube was used. $[\alpha]_D$ is given as

$$X° \pm Y° = \frac{100}{lc}(x° \pm y°),$$

where $x°$ is the difference between the mean of twenty readings with the solution and the mean of twenty readings with a solvent blank; $y°$ is the standard error of this difference. Allowance is thus made for the variation of the polarimeter readings, but not for errors in the weighing of solute or solvent, which are much smaller.

Extraction of urine and preparation of fraction C. Many modifications of the extraction procedure were tried (see Klyne, 1946). The following are details for a batch of urine from which *Z* sulphate was obtained.

Urine (22·2 l., collected in the last 3 weeks before foaling) was extracted with *n*-butanol (4 × 6 l.). Emulsions were broken by allowing to stand with gentle shaking, centrifuging or adding ethanol (1–5% of vol. of urine). The butanol extracts were washed with N-aqueous KOH (4 × 6 l.) and with water (4 × 1·5 l.). Emulsions in the water-washing stage were broken rapidly by the addition of saturated aqueous potassium acetate (10% of vol. of aqueous layer).

The washed butanol extracts were evaporated to dryness *in vacuo* at > 100° and the residue dissolved in water (500 ml.). The aqueous solution so obtained was extracted with ether (4 × 200 ml.) and cooled to 0°; its pH was adjusted to *c.* 2 with HCl and it was again extracted with ether (2 × 300 ml., 4 × 200 ml.). It was then made alkaline to litmus with KOH, warmed to remove ether, cooled, and extracted with butanol (4 × 200 ml.). The butanol extracts were washed with water (3 × 100 ml.) and evaporated to dryness giving the 'crude conjugate fraction' as a brown friable powder (12·3 g.).

This material was dissolved in boiling water (90 ml.), saturated aqueous potassium acetate (90 ml.) was added, the mixture was cooled to 15–20° and the pasty precipitate centrifuged. The precipitate was washed twice by centrifuging with its own volume of half-saturated aqueous potassium acetate and dissolved in moist butanol (250 ml.). This solution was washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate-insoluble fraction' as a yellow powder (8·8 g.).

The supernatant and washings from the potassium acetate precipitation were extracted with butanol (4 × 100 ml.); the extracts were washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate-soluble fraction' as a nearly white powder (2·0 g.).

The 'potassium acetate-insoluble fraction' was dissolved in the minimum volume of boiling water (140 ml.) and cooled to 0°. The sticky precipitate which formed was centrifuged, washed once by centrifuging with its own volume of ice-cold water and dissolved in moist butanol (200 ml.). The butanol solution on evaporation to dryness gave the 'water-insoluble fraction' as a yellow powder (4·7 g.).

The supernatant and washings from the above separation were extracted with butanol (3 × 100 ml.); the extracts were washed with water (3 × 20 ml.) and evaporated to dryness giving the 'water-soluble fraction' as a pale brown powder (3·9 g.).

The 'water-insoluble fraction' was then extracted repeatedly with boiling 98% acetone (2 × 200, 6 × 100 ml.). Each extract was filtered hot and the undissolved solid used for the next extraction. The extracts, which on cooling gave no precipitate, were concentrated on the water bath under a gentle air blast until solid began to separate; on cooling again, a nearly white, partly crystalline precipitate separated (98% acetone solids, fraction *C*; 620 mg.). The melting points of the fractions ranged from 218–219° (decomp.) (first fraction) to 235–240° (decomp.) (last three fractions).

Isolation of Z sulphate as the p*-toluidine salt.* The 98% acetone solids, fraction *C*, from the above separation (301 mg.) were dissolved in boiling water (50 ml.) and a solution of *p*-toluidine hydrochloride (185 mg., 2 equiv.) in water (5 ml.) was added. The copious precipitate which formed was just dissolved by adding more water (35 ml.) at 100°, and the solution was cooled to 0° giving p-*toluidine Z sulphate* (293 mg., m.p. 190–192°). After two recrystallizations from boiling water this salt formed very fine white needles, m.p. 195–197°, after softening at 186°, $[\alpha]_D^{19°} + 31·7° \pm 0·6°$ in chloroform (*c*, 3). Approximate solubilities (in mg./ml.) were as follows: in water, at b.p., 3·5; at 15–20°, 0·5; in chloroform, at 15–20°, >30. The salt showed no loss in weight on drying at 80° *in vacuo*. (Found: C, 67·2, 67·2; H, 8·1, 8·1; N, 2·8, 2·9; S, 6·4, 6·4. $[C_{21}H_{31}OSO_4]^-[CH_3.C_6H_4.NH_3]^+$ requires C, 66·8; H, 8·2; N, 2·8; S, 6·4%.)

Potassium Z sulphate. This was obtained from the 98% acetone solids, fraction *C*, from two batches of urine by repeated crystallization from water and then from ethanol. It was best obtained pure from the *p*-toluidine salt as follows.

p-Toluidine *Z* sulphate (100 mg.) in warm water (100 ml.) was treated with KOH (1·0 g.) in water (5 ml.), and *p*-toluidine was extracted with ether (4 × 25 ml.). The aqueous solution was heated to drive off dissolved ether, cooled, and extracted with butanol (2 × 50, 2 × 25 ml.). The extracts on evaporation gave crude *potassium Z sulphate*, 86 mg. (90% of theory), m.p. 222–224° (decomp.), which was then crystallized from water and from ethanol giving rosettes of needles, m.p. 244–245° (decomp.) after browning at 234° and sintering at 241°.

Analyses of the potassium salt, when compared with those of the *p*-toluidine salt and of the free steroid, showed that it was a *monohydrate*, which did not lose its water of crystallization on drying to constant weight at 80° *in vacuo* over P_2O_5. (Found: C, 55·8, 55·8; H, 7·5, 7·5; K, 8·8, 8·5. $C_{21}H_{31}OSO_4K.H_2O$ requires C, 55·7; H, 7·4; K, 8·6. $C_{21}H_{31}OSO_4K$ requires C, 58·0; H, 7·2; K, 9·0%.)

Approximate solubilities (in mg./ml.) were as follows: in water at b.p., 10–11; at 0°, 3; in ethanol at b.p., 9; at 0°, 2·5; in butanol at b.p., 10; at 0°, 7; in chloroform at b.p. or at 0°, < 0·1.

The potassium salt was dimorphous, clusters of prisms, m.p. 216–217° (decomp.) sometimes appearing when the compound was crystallized from water. (The quantity obtained was insufficient for analysis.) This prismatic form on treatment with *p*-toluidine hydrochloride gave the *p*-toluidine salt described above.

Table 2. *Comparison of Z acetate and* allo*pregn-16-en-3(β)-ol-20-one acetate*

	Z acetate		*allo*Pregnenolone acetate
M.p.	165–166·5°		165–167°*
Mixed m.p.		163–165·5°	
$[\alpha]_D^{22°}$ in chloroform (*c*, 1·4)	+33·9°±0·6°		+36·3°±0·7°*
Ultraviolet absorption†			
$\lambda_{max.}$	240, 320 mμ.		240, 320 mμ.
log $\epsilon_{max.}$	3·95, 1·78		3·93, 1·92*
16:17-*Epoxy compound*			
M.p.	185–187°		183–185°*
Mixed m.p.		183–185°	

* These values were determined on Prof. Plattner's samples in Edinburgh and Liverpool. Plattner *et al.* (1947) give the following values: *allo*pregnenolone acetate, m.p. 166–167°; $[\alpha]_D^{20°}$ +42·2° (*c*, 1·42; chloroform); log $\epsilon_{max.}$ =4·2, 2·1 (solvent not stated). Epoxy compound, m.p. 186–187°.

† In ethanol containing a little ether. We are indebted to Prof. R. A. Morton, Dr A. L. Stubbs and Mr T. W. Goodwin (University of Liverpool) for these measurements.

Compound Z. *p*-Toluidine *Z* sulphate (154 mg.) was dissolved in hot water (100 ml.), 2 N-HCl (100 ml.) was added, and the mixture was heated in the boiling water bath. The initially clear solution became cloudy after 10 min. heating, and a precipitate formed, which gradually increased in bulk. After 2 hr. heating the mixture was cooled and extracted with ether (1 × 100 ml. and 3 × 50 ml.). The ether extracts were washed with N-HCl (2 × 30 ml.), 0·5 M-$NaHCO_3$ (30 ml.) and water (3 × 30 ml.). The extracts on evaporation to dryness yielded crude compound *Z*, 88 mg. (91% of theory), m.p. 197–202° after sintering at 184°. After two recrystallizations from aqueous ethanol, *Z* formed glistening leaflets, m.p. 205–207°, $[\alpha]_D^{17°}$ +50·2°±4·2° in ethanol (*c*, 1·4). (Found: C, 79·5, 79·8; H, 10·3, 10·1. Mol. wt. (Rast) 307, 325. Calc. for $C_{21}H_{32}O_2$: C, 79·7; H, 10·2%. Mol. wt. 316.)

Z acetate. Compound *Z* (7·8 mg.) was heated at 100° for 2 hr. with acetic anhydride (1 ml.) and pyridine (1 ml.). Much water was added, and the white flocculent precipitate obtained was filtered, washed and dried. This material was recrystallized twice from aqueous ethanol (approx. 1 : 1 by vol.) giving pure *Z* acetate in elongated hexagonal leaflets, m.p. 165–166·5°. (Found: C, 76·7, 76·4; H, 9·5, 9·8. Calc. for $C_{23}H_{34}O_3$: C, 77·1; H, 9·6%.)

The epoxy compound of *Z* acetate was prepared by treatment with perbenzoic acid as described by Plattner *et al.* (1947). The properties of *Z* acetate and *allo*pregn-16-en-3(β)-ol-20-one acetate are compared in Table 2.

SUMMARY

1. A general method for the extraction of steroid and other conjugates from mares' urine is described.

2. A new organic sulphate has been isolated from pregnant mares' urine as its *p*-toluidine salt, which on acid hydrolysis yielded *allo*pregn-16-en-3(β)-ol-20-one.

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The Effect of Thyroxine on the Metabolism of Lactating Cows

1. GENERAL RESULTS AND NITROGEN METABOLISM

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That thyroxine causes an increase in milk secretion and in the yield of milk fat has been shown by Graham (1934 *a*, *b*), Jack & Bechdel (1935), Folley & White (1936), Herman, Graham & Turner (1938) and Smith & Dastur (1940). These observations did not at first arouse general interest because of the prohibitive cost of thyroxine. Later, however, Ludwig & von Mutzenbecher (1939) showed that thyroid-active material could be produced from casein by iodination, and this was confirmed by Harington & Pitt-Rivers (1939), who demonstrated that in the iodination thyroxine was formed from the tyrosine of the casein. A few years later the conditions of iodination were further defined by Turner & Reineke (1944) in such a way as to ensure reasonable yields of thyroxine.

Since these observations were made, the use of iodinated casein for increasing the milk yield of cows in commercial herds has been investigated, notably by Blaxter (1945, 1946). Blaxter's work leaves little doubt that, when cows are given iodinated protein in their feed under ordinary conditions of farm management, the extra milk and milk fat produced result not from an increase in the intake of food, but from raised catabolism. The animals lose condition, probably because the extra milk is formed, in part at least, from the proteins and fat of the animals' own tissues during a period when the metabolic rate of the animals is increased. As increased catabolism might have marked adverse effects on lactating cows, the work described in the present paper was designed to determine to what extent the stimulation of milk production by thyroxine is associated with loss of body tissue and with negative nitrogen balance, and whether increase of food intake can compensate for any raised catabolism which may occur. For all the experiments thyroxine was used rather than iodinated protein, because of the risk that the activity of iodinated casein given orally would vary greatly from sample to sample and be difficult to assess. This uncertainty does not exist if a known dose of thyroxine is given subcutaneously.

EXPERIMENTAL

General plan of the experiments and care of the animals. Three experiments were carried out, each involving three Ayrshire cows which, at the beginning of the experiments, were 10–20 weeks from parturition. The animals were kept in special stalls in a metabolism house (Morris & Wright, 1933) in which the urine and faeces excreted by the cows can be collected separately with only slight mutual contamination. Before an experiment began, the cows were given a period of 2 or 3 weeks to accustom themselves to the metabolism house management and to the diets used in the experiment. This was followed by three periods, each lasting for at least 4 weeks. In the first and third periods no thyroxine was given, but in the second period two of the cows were treated with the hormone, the third cow receiving no thyroxine in any of the periods. In this way it was hoped to compare the findings obtained for the thyroxine-treated cows during the second period of each experiment with the findings for the same cows before and after thyroxine treatment, and also with those for the control cow throughout the whole experiment. This was possible for the first two experiments, but during the third experiment the control cow became ill, too late to be replaced, and had to be discarded. All the animals were weighed at the beginning and end of each period. Every 2 days, samples of food, urine, faeces and milk were analyzed.

Diet. In the first experiment the rations allowed per gallon of milk were purposely kept slightly below the lowest level which would ever be likely to be fed in practice. This resulted in negative N balances during the thyroxine period. In the second and third experiments the rationing was made progressively more liberal in order to determine how far the tendency to negative N balance could be overcome by increased food intake.

In the first experiment the maintenance ration consisted of 14 lb. hay and 1 lb. bruised oats/cow/day, and the production ration of 3·13 lb. bruised oats and 0·91 lb. bean meal (*Vicia faba*) per gallon of milk. The amount of production ration fed per day in any week was adjusted according to the yield of milk in the preceding week. The same production ration was fed in the second experiment but at a slightly higher level. In the third experiment the rations contained cubes consisting of wheat feed 42·5%, decorticated groundnut 27·5%, oats 20% and molasses 10%. For the first few weeks of the first control period of this third experiment, the maintenance ration was 14 lb. hay/day, and for production a mixture was made up consisting of equal parts of bruised oats, bean meal and cubes, 4 lb. of the mixture being fed per gallon of milk. Later in this first control period, in order to ensure that the animals would be liberally fed just before and during the thyroxine treatment, the allowance of hay was raised to 16 lb. and the concentrate mixture was changed to oats (3 parts), bean meal (3 parts) and cubes (4 parts), and fed at the rate of 5 lb. per gallon of milk on the basis of the yield during the previous fortnight. This basis of rationing was continued for the remainder of the experiment.

In work of this type, where attempts are made to feed rations strictly according to milk yield, it is not easy to be certain that the intended relationship between milk production and protein intake will be accurately maintained, since quite unpredictable variations in milk yield tend to occur. The best way of ascertaining what the relationship actually was, is to calculate it at the end of the experiment from the figures obtained for the milk yield and for the actual amount of protein eaten. Data obtained in this way for the amount of protein fed per gallon of milk are shown in Table 1.

Table 1. *Protein equivalent ingested (lb./gallon of milk yielded)*

Exp. no.	Cow no.	Period 1 (no thyroxine)	Period 2 (thyroxine)	Period 3 (no thyroxine)
1	1	0·51	0·50	0·77
	2 (control)*	0·44	0·41	0·44
	3	0·52	0·39	0·65
2	4	0·58	0·57	1·00
	5 (control)*	0·53	0·56	0·55
	6	0·50	0·52	0·95
3	7	0·62	0·68	0·87
	8	0·66	0·73	1·03

* The control cows (nos. 2 and 5) received no thyroxine in period 2.

Administration of thyroxine. A solution of 'thyroxine sodium B.P.' (British Drug Houses Ltd.) containing 1 mg./ml. was made up according to Folley & White (1936). Each day during the thyroxine periods 10 ml. of this solution were injected subcutaneously into the flanks of the cows. Cows 1, 3, 4 and 6 were injected for 28 successive days, and cows 7 and 8 for 32 and 31 successive days respectively.

Methods of analysis. Total N was estimated by the Kjeldahl method, the catalyst being K_2SO_4, $CuSO_4$ and powdered selenium. For estimating N in milk, the samples were weighed and not pipetted. Milk fat was estimated by the Gerber process, and milk phosphatase by the method of Kay & Graham (1933). In the first two experiments the amount of urea plus NH_3 in the urine was determined according to Peters & Van Slyke (1932), and in all three experiments the creatine and creatinine contents of urine were estimated by a modification of Jaffe's picric acid method (Hawk & Bergeim, 1927) using a Spekker absorptiometer.

RESULTS

Pulse rates and milk phosphatase. It was essential for the purpose of this investigation to find out as early in the thyroxine period as possible whether the hormone was showing its usual physiological activity. The two criteria used for this purpose were the effect of thyroxine in enhancing the pulse rate and the rapid diminution which thyroxine produces in the concentration of phosphatase in the milk (Folley & White, 1936). Every day, except Sunday, throughout all three experiments pulse rates were determined by palpation of the plantar arteries of a forelimb, care being taken to ensure that the animals were not excited in any way. During the determination the animals were standing up and either eating hay or ruminating just after having eaten it. Four concurrent determinations were made. From the average results (Table 2) it can be seen that with all the treated animals there was a marked increase in pulse rate during the thyroxine period, whereas with the two controls during the same period no significant increase occurred. Table 2 also shows that the time, which elapsed from the first injection to the day when the pulse rate was maximal, varied from cow to cow. With cow 8 the pulse continued to increase during treatment. With all the other treated cows the pulse had passed its maximum before injection ceased.

Averages of many determinations of milk phosphatase are shown in Table 2. With the two controls there was a tendency for the phosphatase in the milk to increase as lactation advanced, whereas with the treated cows there was the expected substantial decrease during hormone treatment.

Table 2. *The effect of thyroxine on the pulse rate and on the phosphatase content of the milk*

		Pulse rate/min.				Phosphatase in milk*		
			Period 2 (thyroxine)					
Exp. no.	Cow no.	Period 1 (no thyroxine)		Days to maximum†	Period 3 (no thyroxine)	Period 1 (no thyroxine)	Period 2 (thyroxine)	Period 3 (no thyroxine)
1	1	46	65	17	49	68	31	84
	2 (control)	51	47	—	46	90	108	111
	3	59	68	6	52	22	7	43
2	4	65	81	19	66	47	8	39
	5 (control)	64	65	—	63	76	110	106
	6	55	60	13	55	39	8	53
3	7	57	90	29	69	136	40	164
	8	62	83	31	61	82	39	154

* Units of Kay & Graham (1933) multiplied by 100.
† Time elapsing from beginning of treatment to the day when the pulse was maximal.

Milk yield. The milk-yield curves for all eight cows are shown in Fig. 1, and the averages for each period in Table 3. It can readily be seen that with the two controls (nos. 2 and 5) the milk yield tended to decrease gradually over the whole period, whereas with the remaining six cows the milk yield was enhanced from about 2 days after hormone injections began until a few days after they ceased. Owing to the difficulty of judging what the yield would have been had no hormone been given, it is not easy to determine quantitatively the effect of thyroxine on milk yield. From the graphs in Fig. 1, however, it has been estimated that the average enhancement of yield by thyroxine was about 30%, which is comparable to results reported by other workers mentioned in the introduction.

Milk composition. With all the treated animals the yield of milk fat was greatly increased during the thyroxine period. Typical results for one control and one treated animal are shown in Fig. 2. With all the treated animals the increase in the yield of fat persisted longer than that of milk as a whole after thyroxine treatment had ceased. The average values for the fat content of the milk and for the nitrogen content of the fat-free milk from all eight cows, and for the non-fatty solids for two of the cows, are

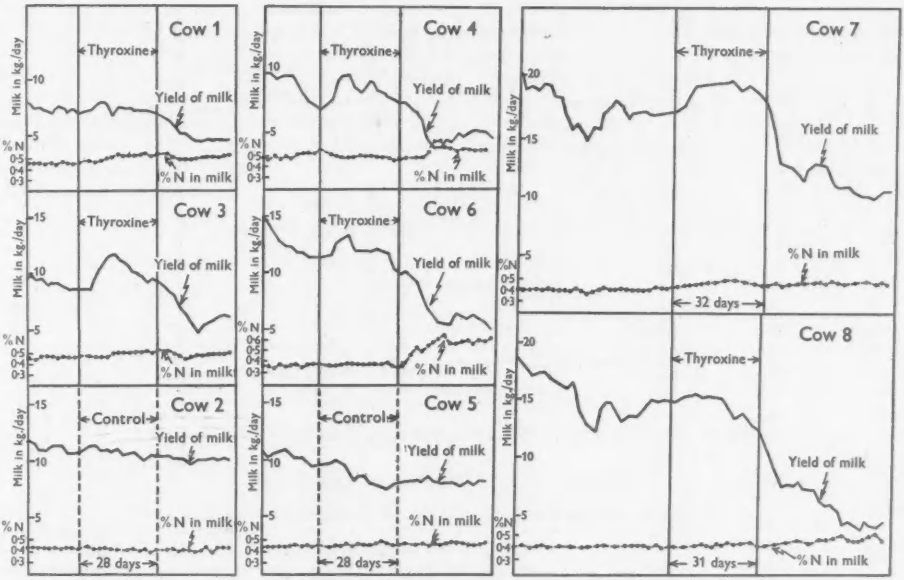

Fig. 1. The effect of thyroxine on milk yield and on the percentage of nitrogen in the milk.

Table 3. *The average yield and composition of the milk produced in each period*

(Thyroxine was given only during period 2 to all cows, with the exception of nos. 2 and 5 which received no hormone at all.)

Exp. no.	Cow no.	Yield of milk (lb./day) Period 1	2	3	Fat content (%) Period 1	2	3	N content of the fat-free milk (%) Period 1	2	3	Solids content of the fat-free milk (%) Period 1	2	3
1	1	7·3	7·3	4·7	4·26	5·65	4·07	0·485	0·529	0·512	—	—	—
	2 (control)	11·2	10·8	10·0	3·63	3·32	3·15	0·451	0·427	0·431	—	—	—
	3	9·3	10·3	6·2	3·79	4·63	3·78	0·484	0·513	0·495	—	—	—
2	4	9·1	8·7	4·7	4·15	4·52	4·38	0·522	0·499	0·563	—	—	—
	5 (control)	10·3	8·5	8·0	2·98	3·10	2·95	0·453	0·477	0·485	—	—	—
	6	12·5	11·6	6·1	4·41	5·19	4·98	0·426	0·504	0·601	—	—	—
3	7	17·6	19·4	12·5	3·56	4·01	3·52	0·434	0·494	0·488	9·11	9·66	9·37
	8	15·2	14·6	6·7	3·70	4·08	4·41	0·441	0·465	0·522	8·77	9·06	9·00

shown in Table 3. Statistical analysis showed that the increases which occurred during hormone treatment were significant.

Body weights. All the animals were weighed at the beginning and end of each period. Each weight recorded was the average of three weighings made at different times on one day (Owen, Smith & Wright, 1943). The weights of the animals at the beginning of the first period of each experiment are shown in Table 4, the changes in weight which occurred during each period being expressed as a percentage of the initial weight. It will be observed that, in contrast to the control cows, all the treated animals lost weight in the thyroxine period, but gained weight again in period 3, after thyroxine injections had ceased. The result of this gain in weight in the final period was that the net loss of weight during the thyroxine and post-thyroxine periods taken together (periods 2 and 3) was of the same order for nearly all the animals whether treated or controls. The only exception was cow 7 which did not show the same degree of recovery in period 3 as the others, although it, like cow 8, was fed the most liberal diet. It should be noted, however, that cow 7 gave a greater increase in the yield of milk fat than cow 8 in the thyroxine period, and that this may have accounted in part for its lower recovery of weight.

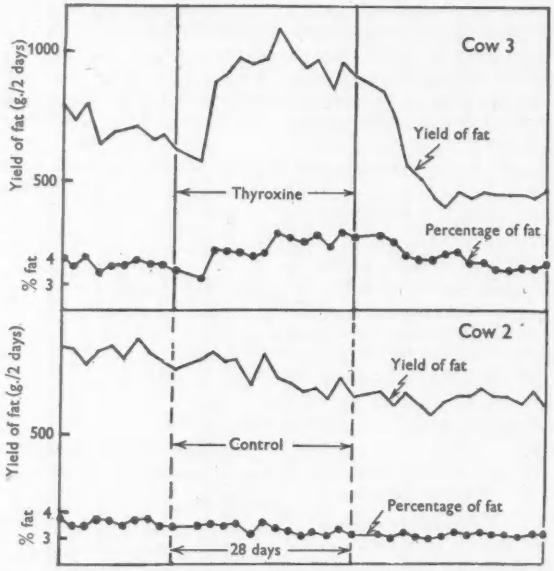

Fig. 2. The effect of thyroxine on the yield and percentage of fat in the milk.

Table 4. *Effect of thyroxine on body weight*

(Thyroxine was given during period 2 to all cows except the controls.)

Exp. no.	Cow no.	Weight at beginning of period 1 (lb.)	Change in weight (lb.) in each period expressed as % of the weight at the beginning of period 1			Change in weight (lb.) in period 2 which was not made good in period 3
			Period 1 (*a*)	Period 2 (*b*)	Period 3 (*c*)	(*b*) + (*c*)
1	1	857	−5·4	− 5·8	+ 4·1	−1·7
	2 (control)	817	0·0	+ 2·1	− 4·2	−2·1
	3	872	−0·4	− 6·9	+ 6·3	−0·6
2	4	823	+0·2	− 9·7	+ 8·7	−1·0
	5 (control)	1006	−0·9	+ 2·0	− 1·3	+0·7
	6	945	−0·9	−13·3	+11·1	−2·2
3	7	1007	+0·2	−10·1	+ 3·2	−6·9
	8	1062	+7·5	− 7·8	+ 6·8	−1·0

As is shown in the next paragraph, the nitrogen balances of cows 7 and 8, unlike those of the other treated cows, did not become negative when the cows were given thyroxine. It is, therefore, difficult to relate the loss in weight occurring during thyroxine treatment to any definite metabolic function. It is probable that the decreases of body weight of cows 1, 3, 4 and 6 were due to loss of both protein and fat, whilst the decreases of body weight shown by cows 7 and 8 were due mainly to loss of fat. Catabolism of fat need not parallel that of protein, while loss of protein from the body is known to be sometimes accompanied by retention of water, as, for example, in famine oedema. With ruminants the percentage of food in the gut is about 12 % (Ritzman & Benedict, 1938), so that changes of 'fill' of the cows may have been partly responsible for changes of weight.

Nitrogen metabolism. All foods, including hay, and also milk, urine and faeces, were analyzed for total nitrogen every 2 days. Average results for each period are recorded in Table 5.

animals, cow 3 showed the greater stimulation of milk yield by thyroxine, and it also showed the greater negative nitrogen balance (−11·5, as compared with −3·5 g. N/day). After hormone treatment ceased, the nitrogen balances of both the experimental animals became markedly positive. In Exp. 2, the results were very similar, in spite of the fact that the cows were allowed a slightly higher protein intake per gallon of milk. In this experiment even the control cow showed a small negative balance of −3·7 g. N/day in period 2. But, whereas the nitrogen balance of this animal remained almost unchanged in period 3, those of cows 4 and 6 became strongly positive (+20·9 and +21·6 g. N/day), after thyroxine treatment had been discontinued. These positive balances in the last period indicate that the retention of nitrogen after thyroxine treatment was much faster than the loss of nitrogen during treatment. In considering the possible effect of the hormone on the cows' health, it is therefore reasonable to suppose that any ill effects due to increased catabolism during hormone treatment may be offset

Table 5. *A summary of the results required to obtain the nitrogen balances*

(Thyroxine was given during period 2 to all cows except the controls. Results are given in g. N/day.)

Exp. no.	Cow no.	Period	Intake*	Output of N in			Total output	Balance
				Milk	Urine	Faeces		
1	1	1	153·4	42·3	31·9	69·4	143·6	+ 9·8
		2	129·1	36·3	32·8	63·5	132·6	− 3·5
		3	126·7	23·2	32·4	54·3	109·9	+16·8
	2 (control)	1	161·1	50·2	29·0	69·4	148·6	+12·5
		2	146·7	44·4	35·1	58·5	138·0	+ 8·7
		3	144·3	41·7	40·2	53·2	135·1	+ 9·2
	3	1	157·2	43·7	25·4	63·1	132·2	+25·0
		2	138·1	50·5	32·9	66·1	149·5	−11·4
		3	135·7	29·6	35·4	56·7	121·7	+14·0
2	4	1	166·3	45·6	40·3	64·8	150·7	+15·6
		2	157·7	41·3	42·0	78·8	162·1	− 4·4
		3	151·9	24·8	37·1	69·1	131·0	+20·9
	5 (control)	1	174·0	45·8	55·7	68·8	170·3	+ 3·7
		2	153·4	39·1	48·4	69·6	157·1	− 3·7
		3	146·2	37·6	44·5	67·3	149·4	− 3·2
	6	1	190·0	59·7	58·4	70·7	188·8	+ 1·2
		2	181·2	55·4	55·7	79·7	190·8	− 9·6
		3	176·4	34·7	49·4	70·7	154·8	+21·6
3	7	1	277·2	74·4	71·9	112·0	258·3	+18·9
		2	315·7	93·0	95·9	120·6	309·5	+ 6·2
		3	250·5	56·4	64·5	108·4	229·3	+21·2
	8	1	256·7	66·6	92·3	93·3	252·2	+ 4·5
		2	261·1	64·2	86·6	102·3	253·1	+ 8·0
		3	172·6	30·6	58·3	79·5	168·4	+ 4·2

* Corrected for food refusals which occurred but seldom and were very small.

In Exp. 1, during the thyroxine period, the nitrogen balances for both the treated cows became negative, whereas the nitrogen balance in the control cow remained positive. Of the two treated by the raised anabolism after the treatment is discontinued.

In Exp. 3, in which the allowance of protein per gallon of milk was further increased, the two cows

7 and 8 showed increases in the yields of milk and milk fat which were very similar to those of cow 6 in Exp. 2, and yet their nitrogen balances remained positive throughout the entire experiment. The positive balance for cow 7 was reduced during thyroxine treatment, but increased again after treatment ceased. But with cow 8, which showed a much larger stimulation of milk production than any of the other cows, the positive nitrogen balance was slightly increased during hormone treatment. It may be concluded from these findings that, on normal rations, the increased milk yield produced by 10 mg. thyroxine/day is accompanied by an accelerated protein catabolism, but that this excessive catabolism may be inhibited by increasing the intake of food.

Partition of urinary nitrogen

(1) *Urea plus ammonia.* The proportion of urinary nitrogen excreted as urea plus ammonia was determined for the composite 2-day samples of urine collected from all six cows during the first two experiments. The results showed a constant ratio (60%) of urea plus ammonia to total nitrogen throughout all three periods.

(2) *Creatine and creatinine.* The results for the excretion of creatine and creatinine in Exp. 2 are shown in Fig. 3. The corresponding graphs for the first and third experiments were very similar. No significant change was noted in the creatinine excretion for any of the cows, nor in the creatine excretion of the control animals (e.g. cow 5, Fig. 3). With the treated animals, however, changes in the creatine excretion tended to follow changes in milk yield. Thus, in the thyroxine period, the creatine excretion was first enhanced, and then began to fall some time before thyroxine treatment ceased. After treatment ceased creatine excretion fell rapidly to a very low value, the fall corresponding roughly to that in milk yield. Like the milk yield, the creatine output then increased again. In the third experiment, where the rations were much more liberal and where the nitrogen balance was always positive, the creatine excretion was again maximal during thyroxine treatment and minimal immediately after the treatment ceased, but the changes were smaller with these two cows (particularly with cow 8) than with the animals in the first two experiments in which the nitrogen balances became negative during treatment. If it is supposed that the creatine is of catabolic origin, these variations in the amount excreted are just what would be expected. Stimulation of milk yield might well be associated with increased catabolism, and this would be accompanied by an increased output of creatine. It does not follow, however, that creatine excretion is causally related to milk production, for it is probable that the creatinuria occurring during hormone treatment is merely a result of the general increase in metabolic rate which thyroxine produces in the tissues as a whole.

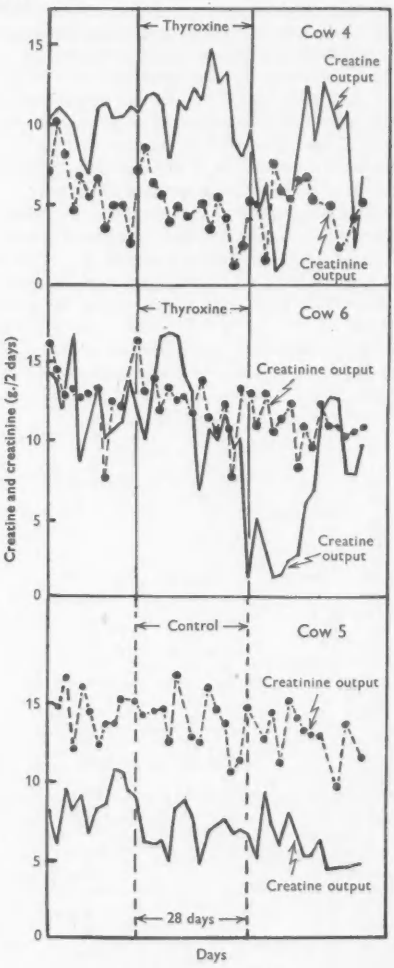

Fig. 3. The effect of thyroxine on the output of creatine and creatinine in the urine.

For ease in comparing the excretion of creatine and creatinine nitrogen, the results have been recorded in Table 6 as percentages of the total urinary nitrogen. The creatinine figures were variable and showed no consistent change which could be related to the hormone treatment. As would be expected from the creatine outputs (Fig. 3), and from the urinary nitrogen outputs (Table 5), the creatine figures for the treated animals tended to

be slightly higher during hormone treatment than before it. When thyroxine administration was discontinued, creatine output decreased. This was marked with cows 1, 3, 4 and 6, but not so marked with cows 7 and 8. The same tendency was noted for control cow 2, but not for control cow 5. This control cow 2 there was a reasonably close parallelism between the volume and the amount of urinary nitrogen throughout the pre-thyroxine and thyroxine periods. This parallelism also existed with cows 1 and 3 before hormone treatment, but, some days after treatment began, a substantial increase

Table 6. *Output of creatine and creatinine (expressed as percentage of total urinary nitrogen*

		Creatine Period			Creatinine Period		
Exp. no.	Cow no.	1	2	3	1	2	3
1	1	9·5	10·5	6·6	4·0	4·4	3·5
	2 (control)	6·1	6·7	5·5	6·4	6·4	5·1
	3	6·8	8·8	4·6	11·1	8·2	7·2
2	4	7·1	7·6	5·4	4·2	3·1	3·9
	5 (control)	4·4	4·1	4·4	7·1	8·0	7·8
	6	5·9	5·8	3·7	6·2	6·1	6·2
3	7	2·3	2·4	2·0	2·9	3·3	3·6
	8	1·0	1·5	1·1	3·4	4·5	5·1

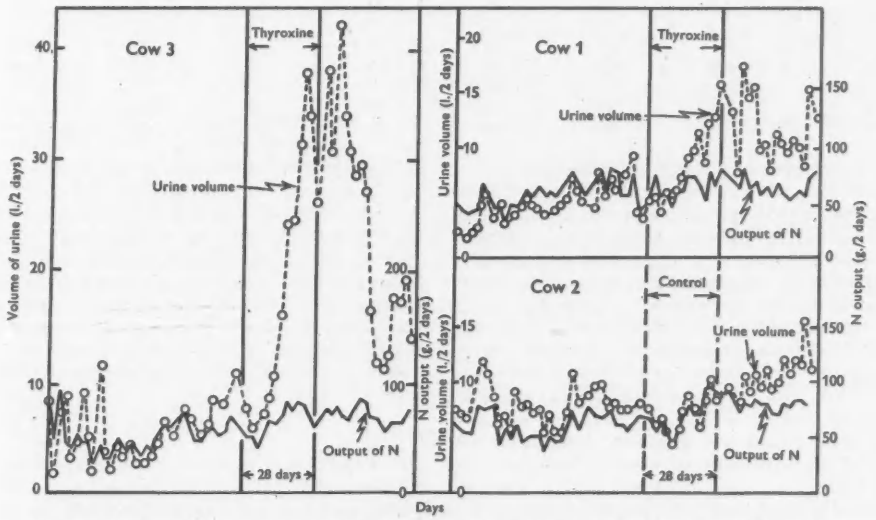

Fig. 4. The effect of thyroxine on the volume of urine and on the output of total nitrogen in the urine.

difference between the control cows may be related to the fact that cow 5 was fed at a slightly higher level than cow 2. For the best fed cows (7 and 8) the percentage of total urinary nitrogen excreted, as creatine and creatinine, was much smaller than for the other cows. This was due to the fact that the total amount of nitrogen excreted in the urine was naturally higher for the best-fed animals than for the others (Table 5).

The diuretic effect

The volume of urine and the urinary nitrogen excreted per day by the cows in the first experiment are shown in Fig. 4. It will be noted that for the in volume occurred. An increase in volume was shown at this stage by all the experimental cows, but not by either of the controls. This may have been caused in part by the cows using water to disperse some of the extra heat generated in their tissues when thyroxine increased the metabolic rate, or it may be that thyroxine raised the activity of the kidneys either directly or by stimulating the secretion of a diuretic hormone. The very large increase in urinary volume noted for cow 3 (Fig. 4) was exceptional, the increase in volume with the other experimental animals being of the order of that shown for cow 1. It is not known why the increase with cow 3 was so large.

The effect of thyroxine on the utilization of food

Apparent digestibility of dietary nitrogen. An attempt is made to assess the effect of thyroxine on the utilization of food by calculating the average values for apparent digestibility of the dietary nitrogen and the efficiency of its conversion to milk nitrogen in each of the three periods (Table 7). In Exp. 1 the apparent digestibility with control cow 2 increased as the experiment progressed, whereas with the treated animals there was a substantial decrease during thyroxine treatment. In Exp. 2 the control animal (no. 5) showed a progressive decrease in the apparent digestibility of its dietary nitrogen. The treated animals, on the other hand, like the treated animals in the first experiment, showed a decrease during hormone treatment, which changed to an increase when hormone treatment ceased. In Exp. 3 there was no significant difference in the results for periods 1 and 2, but when thyroxine treatment ceased the apparent digestibility decreased. This difference between the changes noted in the first two experiments and those in the third is probably associated with the much higher level of feeding in the last experiment. It may be concluded that at the lower levels of feeding the apparent digestibility of the dietary nitrogen tends to be decreased when thyroxine is administered. Estimation of the moisture content of the faeces throughout the entire experiment showed that during thyroxine treatment the faeces were more moist. They were also obviously softer and more liquid than in the control periods. This might tend to cause the contents of the alimentary tract to be retained for a shorter time during thyroxine treatment than is usually the case, and this, in turn, might cause a reduction in the amount of nitrogen absorbed. With cows 7 and 8 the more rapid passage of food caused by thyroxine treatment would tend to offset the effect of the more liberal feeding.

Conversion of dietary nitrogen to milk nitrogen. The results in Table 7 show that in the first two experiments thyroxine caused a marked increase in the apparent efficiency with which the dietary nitrogen was converted to milk nitrogen. When hormone treatment ceased an even more marked change, in this case a decrease in apparent efficiency, occurred. Of the two control cows (2 and 5) one behaved differently from the other in this respect. The efficiency of conversion decreased with cow 2 and increased with cow 5 as the experiment progressed, but they both differed from the experimental animals in not showing a maximum in period 2. In Exp. 3 the better fed cows (7 and 8) did not show any significant change in efficiency of conversion when thyroxine was given, in spite of the larger increases in milk yield. They also showed smaller decreases in efficiency of conversion when thyroxine was discontinued.

Table 7. *The apparent digestibility of the dietary nitrogen and the efficiency of conversion of the dietary nitrogen to milk nitrogen*

(The apparent digestibility is defined as $100\,\frac{(\text{Intake N} - \text{faecal N})}{\text{Intake N}}$; the conversion of dietary N to milk N is expressed as the ratio $\frac{\text{Milk N} \times 100}{\text{Intake N} - \text{faecal N}}$.)

		Apparent digestibility Period			Conversion of dietary N to milk N Period		
Exp. no.	Cow no.	1	2	3	1	2	3
1	1	55	51	57	50	55	32
	2 (control)	57	60	63	65	50	46
	3	60	52	58	46	71	38
2	4	61	50	55	45	52	30
	5 (control)	61	55	54	44	47	48
	6	63	56	60	50	55	33
3	7	59	62	57	51	50	47
	8	64	60	54	39	40	33

DISCUSSION

The experiments reported show that, under conditions of controlled food intake, the effect of thyroxine in stimulating the yield of milk can be attributed mainly to an increase in catabolism. The metabolic rate, as measured by the pulse rate, is much accelerated, so that weight is lost and nitrogen balance becomes negative. However, negative nitrogen balances during thyroxine treatment can be inhibited by a more liberal allowance of food. This diminution of catabolism as a result of increasing food intake recalls a similar phenomenon, reported by Cuthbertson (1942), Croft & Peters (1945) and Taylor (1943), who found that large negative nitrogen balances resulting from trauma could be reversed by increasing the food intake.

The effects of thyroxine in increasing catabolism were reversed during the period following its discontinuance. Nitrogen retention increased markedly, the pulse rate reverted to somewhat less than its

pretreatment level, and the graphs for the yields of milk and milk fat showed distinct minima. During this last period of the experiments tissue anabolism evidently took precedence over milk secretion in its claim for available nutrients.

The foodstuffs used in these experiments were, with the exception of groundnut, such as can readily be grown in Britain. It might, therefore, at times be economic to obtain a temporary increase of the milk supply by the feeding of iodinated casein, the action of which is very similar to that of thyroxine. Although such feeding would temporarily increase catabolism, any consequent ill effects would be largely overcome by the stimulus to anabolism which occurs when the treatment is discontinued. Any subsequent loss of efficiency by the cow would have to be balanced against the temporary gain of milk yield without any increase in the number of cows in the herd, in the man-hours or in the materials other than foodstuffs required for its production.

SUMMARY

1. The effect of injecting 10 mg. thyroxine/day for 4 weeks into cows at three different levels of food intake has been investigated. The resultant increase in milk yield was accompanied by an increase in pulse rate, by loss of weight, by markedly negative nitrogen balance and by an increase in the excretion of water and creatine in the urine. The percentage of fat in the milk was increased and small but statistically significant increases were observed in the percentage of solids and of nitrogen in the fat-free milk.

2. Thyroxine appeared to stimulate lactation by increasing the cows' catabolism, and this resulted in negative nitrogen balances.

3. The negative nitrogen balances could be inhibited by increasing the intake of food.

The author wishes to thank members of the staff of the Hannah Institute for help in these experiments. Thanks are particularly due to Dr N. C. Wright for suggesting the investigation, and to Dr J. A. B. Smith for his collaboration.

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The Effect of Thyroxine on the Metabolism of Lactating Cows

2. CALCIUM AND PHOSPHORUS METABOLISM

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In the previous paper (Owen, 1948), experiments have been described on the effect of thyroxine injections on the nitrogen metabolism of lactating cows. During these experiments, data for the calculation of calcium balances were obtained for all the cows, and data for phosphorus balances for two of them. The purpose of the present communication is to report the effect of thyroxine on the calcium and phosphorus contents of the milk, and also on the calcium and phosphorus metabolism of the cows when thyroxine was administered with the object of increasing the milk yield.

EXPERIMENTAL

The general plan of the experiments and the nature of the rations have been described already (Owen, 1948). There were three experiments, each comprising three periods. The intention was to have three cows in each experiment, but in

the last experiment only two of the three completed the required period in the metabolism house. There were, therefore, eight animals in all. In the first and third periods of each experiment, no thyroxine was given, but in the second period two of the cows were injected with 10 mg. thyroxine daily for at least 28 days. The third cow acted as a control and received no thyroxine in any of the periods. For all eight animals Ca was determined in the food, milk, urine and faeces, and from these data the Ca balances were calculated for each period. For the two animals in the third experiment the same was done for P.

Methods. Owing to the amount of work involved in metabolism studies of this type with large animals, the Ca and P analyses had to be carried out after the main experimental work was finished. It was necessary, therefore, to store samples of the food, milk, urine and faeces. Milk samples were preserved with formaldehyde, faeces were stored dry in stoppered bottles and urine samples were kept in a refrigerator. Before analysis the urine samples were made acid to dimethylaminoazobenzene (Töpfer's reagent, pH 2·9) by additions of 6 N-HCl. By this means any deposits of Ca and phosphate were dissolved, and copious precipitation of uric acid occurred. Analysis of this precipitate, however, showed that it did not contain any Ca or P. Every second 2-day sample of food, milk and excreta was analyzed.

Analytical methods used were identical with those employed previously in human balance experiments (Owen, 1939). The samples were ashed in vitreosil basins in an electric muffle furnace which was thermostatically controlled so that its temperature did not exceed 600°. 10% $Mg(NO_3)_2$ was added to food, milk and faeces to prevent loss of P by volatilization, and the samples were dried on a sand bath before being placed in the muffle furnace. The ash was dissolved in warm 6 N-HCl and made up to a known volume. After silica had been allowed to settle overnight, samples were taken for analysis. With urine it was found impracticable to estimate Ca and P on the same sample of ash. This was because, on using $Mg(NO_3)_2$ to bind the P, serious deflagration occurred when the mixture was dried, whilst with $MgCl_2$, the resulting glassy ash caused the basins to crack on cooling. For estimating P, therefore, the urine was digested with H_2SO_4 and 30% H_2O_2 (Horecker, Ma & Haas, 1940), and for the estimation of Ca the urine was ashed in the muffle furnace without addition of magnesium salts. The final stage in the estimation of calcium was the titration of Ca oxalate with $KMnO_4$. In the P estimation the strengths of the coloured solutions were determined in a Spekker absorptiometer.

RESULTS

Milk composition. The average figures for the content of calcium and phosphorus in the fat-free milk are shown in Table 1. Inspection and statistical analysis of these results showed that the variations which occurred in the calcium content could not be attributed to the administration of thyroxine. Thus, with cows 1, 3, 6 and 7, there was a slight increase in the calcium content during thyroxine treatment as compared with the preceding period; whereas, with cows 4 and 8, there was a slight decrease. Of the control cows, no. 2 showed a slight decrease and no. 5 a slight increase in calcium content during period 2 as compared with period 1.

Table 1. *The calcium and phosphorus contents of the fat-free milk before, during and after thyroxine treatment*

(Thyroxine was given during period 2, except to the control cows 2 and 5.)

		Calcium (g./100 ml.) Period			Phosphorus (g./100 ml.) Period		
Exp. no.	Cow no.	1	2	3	1	2	3
1	1	0·104	0·115	0·130	—	—	—
	2 (control)	0·114	0·109	0·127	—	—	—
	3	0·107	0·115	0·117	—	—	—
2	4	0·125	0·119	0·125	—	—	—
	5 (control)	0·090	0·094	0·095	—	—	—
	6	0·130	0·138	0·142	—	—	—
3	7	0·128	0·142	0·130	0·0945	0·1089	0·0966
	8	0·141	0·133	0·134	0·0946	0·1106	0·1008

The phosphorus results were obtained for two cows only, but with both of them, there was a marked increase in the phosphorus content of the fat-free milk during thyroxine treatment; and after treatment ceased the values tended to return towards their pre-thyroxine level. The increases of 15 and 17%, which occurred during hormone treatment as compared with the pre-thyroxine level, were statistically significant.

Calcium metabolism. During all periods of the experiments (Table 2), the cows were in negative calcium balance, except for cow 4 which showed a small positive balance of 0·9 g. Ca/day in the period after thyroxine treatment ceased. The type of fluctuation occurring from one 4-day period to another, as found in Exp. 3, can be seen from Fig. 1. It is clear that the output of calcium in the urine did not vary appreciably, and that it was not affected by thyroxine treatment.

It will be noted that a minimum occurred in the graph of calcium intake (Fig. 1). This resulted from the fact that the chief source of calcium in the diet was the hay, and during that particular period of Exp. 3 the calcium content of the hay was low. The apparent digestibility of the calcium is shown in Table 2. The values became negative in periods 2 and 3 of the third experiment.

Table 2. *The apparent digestibility of calcium and a summary of the results required to obtain the calcium balances*

(Thyroxine was given during period 2, except to the control cows 2 and 5. All results which are average figures are given in g. Ca/day.)

Exp. no.	Cow no.	Period	Ca intake	Output of Ca: Milk	Urine	Faeces	Total output	Balance	Apparent* digestibility of Ca
1	1	1	14·6	9·6	0·6	11·1	21·3	− 6·7	24·1
		2	14·6	8·3	0·4	12·5	21·2	− 6·4	15·0
		3	14·6	5·1	0·9	11·9	17·9	− 3·3	19·0
	2 (control)	1	15·2	13·2	0·9	11·0	25·1	− 9·9	27·8
		2	15·2	11·8	0·8	9·1	21·7	− 6·5	40·5
		3	15·2	11·5	0·6	9·8	21·9	− 6·7	35·6
	3	1	14·9	12·4	1·2	9·4	23·0	− 8·1	37·2
		2	14·9	13·1	1·8	9·1	24·0	− 9·1	38·9
		3	14·9	7·3	2·6	7·3	17·2	− 2·3	51·0
2	4	1	18·3	10·4	0·7	12·8	23·9	− 5·6	30·4
		2	19·3	10·0	1·1	16·0	27·1	− 7·8	16·9
		3	19·2	5·5	1·2	11·6	18·3	+ 0·9	39·8
	5 (control)	1	19·6	8·9	1·5	12·7	23·1	− 3·5	34·8
		2	19·1	7·6	2·0	14·4	24·0	− 4·9	24·6
		3	18·9	7·4	2·0	13·9	23·3	− 4·4	26·8
	6	1	20·3	14·8	1·5	13·5	29·8	− 9·5	33·5
		2	20·3	15·4	0·7	14·2	30·3	−10·0	29·7
		3	20·2	8·2	1·2	13·2	22·6	− 2·4	35·0
3	7	1	41·0	21·6	1·8	33·8	57·2	−16·2	17·2
		2	40·0	26·8	1·5	40·9	69·2	−29·1	− 2·2
		3	31·4	15·1	1·5	36·8	53·4	−22·0	−17·2
	8	1	39·4	20·6	1·8	31·4	53·8	−14·4	20·3
		2	36·2	18·3	1·2	38·7	58·2	−22·0	− 6·9
		3	25·6	7·5	1·7	35·1	44·3	−18·7	−37·1

* $100 \frac{(\text{Intake of Ca} - \text{faecal Ca})}{\text{Intake of Ca}}$.

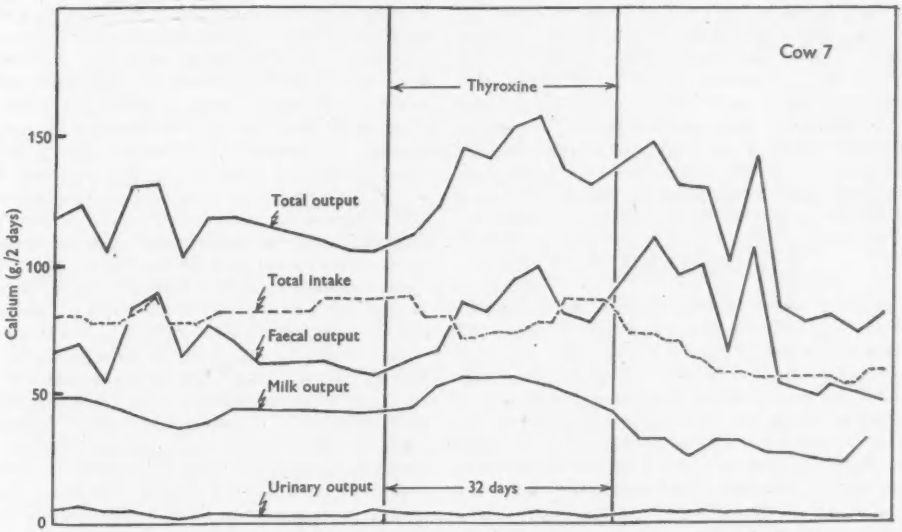

Fig. 1. The effect of thyroxine on the output of calcium in milk, urine and faeces. The calcium intake is also shown.

Phosphorus metabolism. The data obtained for calculating the phosphorus balances for cows 7 and 8 are shown in Table 3. The balances were all positive throughout the whole of Exp. 3, and in this respect they resembled the nitrogen balances rather than the calcium balances.

Table 3. *The apparent digestibility of phosphorus and a summary of the results required to obtain the phosphorus balances in Exp.* 3

(Thyroxine was given during period 2. All results which are average figures are given in g. P/day.)

Cow no.	Period	Intake of P	Output of P			Total output	Balance	Apparent* digestibility of P
			Milk	Urine	Faeces			
7	1	51·3	15·8	0·25	30·5	46·6	+ 4·7	40·6
	2	66·2	20·1	0·62	35·4	56·1	+10·1	46·6
	3	51·2	11·1	0·41	31·6	43·1	+ 8·1	38·3
8	1	45·7	14·2	0·18	27·3	41·7	+ 4·0	40·3
	2	55·0	15·0	0·58	29·3	44·9	+10·1	46·7
	3	35·9	5·8	0·44	23·4	29·6	+ 6·3	34·8

$$* \; 100 \frac{(\text{Intake of P} - \text{faecal P})}{\text{Intake of P}}.$$

The outputs of phosphorus in the milk, faeces and urine were all greater in the thyroxine period than in the periods preceding and following hormone treatment. Unlike the corresponding results for calcium, the urinary output of phosphorus was very markedly increased, but this made little difference to the total excretion since the proportion of the output accountable to the urine was very small. In spite of the fact that the output of phosphorus was increased during the thyroxine period, the balances showed that the amount of phosphorus retained by the cows was also greatest in that period. It was possible to have increases occurring simultaneously in both output and retention in period 2, since during that period extra rations were fed according to the increase in milk production, and this led to a greater intake of phosphorus. The apparent digestibility of the phosphorus (Table 3), unlike that of calcium (Table 2), was higher during the thyroxine period than in the other two periods.

DISCUSSION

Negative calcium balances in lactating animals in early or mid-lactation are quite commonly observed (Mackintosh, 1938; Garry & Wood, 1946). It is not surprising to find, therefore, that with the animals used in the present work, the calcium balances were almost all negative, the only exception being the value for cow 4 in the third period of Exp. 2 which was slightly positive. In the first two experiments the balance in the first period was on the whole very similar to that during thyroxine treatment, but a big decrease in negative balance occurred after thyroxine administration had ceased. This can be attributed largely to the decrease in milk yield, which resulted in a diminished output of calcium in the milk during the third period. In the third experiment, the negative balances were more marked during thyroxine treatment than either before or after treatment. This was due mainly to an increase in the output of faecal calcium during the thyroxine period. Indeed, it can be seen from Fig. 1 that faecal output of calcium was closely paralleled by total output. Before these results were obtained, it was expected, from the work of Hunter (1930) on humans, that thyroxine treatment would cause all the cows to exhibit a marked increase in the amount of calcium excreted in the urine. This, however, did not occur. Owing to the bulkiness and relative indigestibility of their food as compared with that of non-vegetarian animals, cows void more water in their faeces than in their urine, and also, their urine tends to be more alkaline. Possibly it is due to both these facts that the faecal but not the urinary excretions of calcium were increased. The absence of effect of thyroxine on the urinary excretion of calcium, and its effect on the faecal calcium, is well shown with cow 7 in Fig. 1. The corresponding graphs for cow 8 were very similar. The only cow which showed an increased output of urinary calcium during thyroxine treatment, the increase continuing after thyroxine injections ceased, was cow 3, and it was this cow which excreted an exceptionally large volume of urine in the latter part of period 2 and the early part of period 3 (Owen, 1948).

The results for the apparent digestibility of the dietary calcium, which are shown in Table 2, are very low. Indeed with cows 7 and 8, negative values were obtained in the second and third periods of Exp. 3, more calcium having been voided in the faeces than had been taken in in the food. Hunter (1930) records that Aub observed this same phenomenon in humans. In these two cows, therefore, thyroxine must have brought about a large increase in the production of faecal metabolic calcium, and

even after thyroxine treatment had ceased, the excretion of calcium relative to the amount taken in still remained very high.

These negative calcium balances raise the question as to whether cows on winter rations, to whom thyroxine is being administered either as the hormone itself or as iodinated casein, should be given some additional source of readily assimilable calcium. Even if negative calcium balances could not be entirely prevented by dietary measures, it is reasonable to expect that extra dietary calcium would help to replace the loss engendered by increased excretion in the faeces. If this extra loss of calcium in cows treated with thyroxine were not replaced, the cows might have to start their next lactation with depleted stores of mobilizable calcium. Progressive chronic depletion of the skeleton by successive reproductive cycles of pregnancy and lactation on diets containing suboptimal amounts of calcium has been recorded in so many species of animals by many observers, that an extra drain caused by the administration of thyroxine should not be allowed to go uncompensated. Some source of extra readily assimilable calcium such as steamed bone flour or defluorinated rock phosphate, or fish meal, could with advantage be included in the rations of any cow to which iodinated casein or other source of exogenous thyroxine is to be fed.

Since some 99 % of the calcium and some 70–80 % of phosphorus in the animal body are in the skeleton (McCollum, Orent-Keiles & Day, 1939), it is perhaps surprising to find that the phosphorus metabolism was so different from that of the calcium. The phosphorus balances in Table 3 were all positive, and they were more strongly positive in the thyroxine period than in either of the other two periods. Unlike that of the calcium, the apparent digestibility of the phosphorus was increased during thyroxine treatment.

It would appear from these results that phosphorus deficiency is not likely to occur in cows which are receiving thyroxine or iodinated casein, but this conclusion can be put forward only tentatively since it is realized that the results on which it is based were obtained with only two cows.

SUMMARY

1. Eight Ayrshire cows in mid-lactation were used to determine the effect of thyroxine administration on the metabolism of calcium and phosphorus. With one exception, the calcium balances were negative both for the treated and for the control animals throughout all the experiments.

2. With two of the cows which were fed more liberally than the others, the output of calcium was greatest in the thyroxine period, and this was attributable to an increase in the faecal metabolic calcium resulting from the hormone treatment. There was no increased output of calcium in the urine.

3. The apparent digestibility of the dietary calcium was found to be very low. This, together with the negative balances, might well result in thyroxine treatment leading to a depletion of calcium reserves which would be dangerously excessive unless dietary measures were taken to make good the loss.

4. Unlike those of calcium, the phosphorus balances (obtained for two cows only) were positive throughout the whole experiment, thyroxine actually tending to increase the amount of phosphorus retained.

5. The calcium content of the milk was not affected by thyroxine, but the phosphorus content was significantly increased.

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Lignins in Young Plants

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In contrast to the extensive research into the properties and structure of the wood lignins, little work has been done on the lignins which occur in the cell walls of annual plants in considerable quantities. These amount to as much as 10–20% of the dry matter in the common forage plants, according to the analytical methods usually employed, which use concentrated mineral acids for lignin determinations. Degradation experiments have been carried out only with lignins contained in the more woody tissues of this kind, such as maize cobs or various straws, where a closer similarity to the wood lignins exists (Phillips, 1944). The lignins of young green plants seem to be digestible to a certain extent (Juon, 1934; Crampton, 1939; McAnally, 1942; Bondi & Meyer, 1943), whereas lignins in straw or maize cobs were found to be practically indigestible (König & Fürstenberg, 1906–7; Thomann, 1921; Rogozinski & Starzewska, 1929; Ferguson, 1942).

Lignins from young plants and from animal faeces have, to our knowledge, not been prepared in pure form. This paper presents an attempt to isolate lignins in a state of comparative purity from a number of young plants, and to investigate their chemical properties in order to compare them with the better-known wood lignins. Furthermore, lignins were prepared from the faeces of sheep which had been given the same plants as their sole food. This procedure allowed us to follow the chemical changes undergone by the lignins in the body of a typical ruminant.

EXPERIMENTAL

Materials

All the plants used for lignin extraction (and fed to sheep for obtaining faeces) were annuals, as follows, the age at cutting being given in parentheses:

Eragrostis tef (2·5 months), *Pencillaria* (2·5 months), *Setaria italica* (2·5 months), and *Hordeum murinum*, wild barley (3·5 months) represent the Gramineae.

Trifolium alexandrinum bersem, Bersem clover (1·5 months), *T. alexandrinum fahli*, Fahli clover (2·5 months), *Arachis hypogea*, peanut hay (4 months), and *Lathyrus ochrus*, Cyprus vetch (2 months) represent the Leguminosae.

Plants belonging to these two families were chosen because they show characteristic differences. Leguminosae remain comparatively soft and non-ligneous until the last stages of their development, while in the Gramineae woodiness sets in early, especially in the stalks. None of the plants used in this investigation was very woody. Peanut hay and wild barley were the materials in which woodiness had developed most.

The plant material was divided into two parts: one was dried and the lignin subsequently extracted, the other part was fed to sheep. In accordance with the usual procedure in digestibility experiments (see Bondi & Meyer, 1940), the various plants were in each case given to the sheep, kept in metabolism cages, as their only food. After a preliminary period of 6 days, faeces of the sheep were collected during the following experimental period of 10 days. From the dried sheep faeces, lignin was extracted as in the case of the plants.

Extraction methods

The separation of lignin from the other components of the plant can be achieved in two ways. One is to dissolve out all materials other than lignin, and leave it as the only remaining material. In the second method the lignin is dissolved out of the plant tissues leaving the other materials. The first method requires the action of concentrated mineral acids on the plant material. In our experience, however, this method is not suitable for the preparation of larger quantities of lignin from green plants, since the action of strong acids may cause the condensation of soluble carbohydrates contained in them, giving black insoluble substances often erroneously classified as 'lignins' (see Hilpert & Hellwage, 1935).

The second type of methods for the isolation of the lignin includes those in which lignin is extracted by the action of organic solvents such as ethanolic HCl or phenol, or by alkali. We did not use organic solvents for extraction, since these bring about condensation reactions or even degradation of the lignin. Instead, we had recourse to the extraction of lignin by means of alkali. At first we used the method proposed by Phillips & Goss (1934) for the extraction of lignin from maize cobs. They extracted the lignin with 60% ethanol containing NaOH (2% w/w) and, after evaporating the ethanol, precipitated it with an excess of ice-cold 0·4N-H_2SO_4. The yields of lignins obtained from green plants by these methods were very small: 0·8–1·6% of lignin were obtained from plants, and 0·9–1·8% from faeces. These figures are much smaller than those obtained in the analytical determination of lignin by treatment with concentrated acids, which gives values of 10–15% in the same forage plants and faecal matter. The lignin obtained by Phillips's method also contains 1·1–3·2% N, according to the type of forage plant. Neither an increase of the

concentration of the ethanol in the extracting solution (up to 96 %), nor an increase in the concentration of NaOH (up to 10 %), brought about either a noteworthy increase in the lignin yield or a decrease in the nitrogen content of the lignin. The raising of the ethanol concentration in the extraction liquid brought about a decrease in the lignin yield.

Since, in spite of the varying of the experimental conditions, the extraction of lignin with ethanolic NaOH did not give practical yields, another method was worked out using hot aqueous 0·5 N-NaOH (see also Phillips, 1929). By this method lignins are extracted together with considerable amounts of polysaccharides, probably hemicelluloses. Both materials are precipitated by acidifying the alkaline extract. The lignins are separated from the hemicellulose by redissolving the precipitate in alkali and adding a large excess of slightly acidified ethanol, which dissolves the lignins, leaving the hemicelluloses. The ethanol-soluble lignins still contain about 20 % of hemicellulose, which can, however, be removed easily by a mild acid hydrolysis. The experimental details are as follows:

The plant or faecal material is ground coarsely, extracted thoroughly with ether and dried. 1 kg. of this material and 12 l. of 0·5 N-NaOH are put in a vessel of about 16 l. capacity (to avoid foaming over), and heated for 8 hr. to 80–85°, with continuous stirring. The entire contents of the vessel are filtered through cheese cloth, and the residue is washed several times with water (5–6 l. in all). The washings are combined with the filtrate, while the residue is extracted three more times under the same conditions. The extracts are combined and evaporated *in vacuo* to about one third of their original volume. After cooling, the residue is acidified with 5 N-H_2SO_4. This precipitates the lignin (together with considerable amounts of hemicellulose) which is allowed to settle out, after which the supernatant liquid is decanted. The precipitate is washed several times with water, which is decanted off each time, until the supernatant liquid appears only slightly yellow. The precipitate is then separated by centrifuging at 2000 r.p.m. for 15 min., or by filtration.

In order to purify the crude product it is again dissolved in the smallest possible quantity of warm aqueous 1·25 N-NaOH. This solution is added to 4 vol. of 96 % ethanol and acidified slightly. The dark-brown supernatant liquid which contains the lignin is separated from the residue (1) by filtration or centrifuging. The greater part of the ethanol is then driven off; this causes precipitation of the lignin. The resulting aqueous suspension of lignin is treated with enough NaOH to dissolve all the lignin, and then 5 N-H_2SO_4 is added to make it just acid, causing separation of the lignin in voluminous flakes. The lignin is difficult to filter and contains varying amounts of hemicellulose. In order to remove the latter, the acidified suspension is heated to boiling. At this temperature hydrolysis of the hemicellulose and coagulation of the lignin take place, and the lignin precipitate becomes coarse and easily filtrable. The impurities remain in the solution, which is usually deep-brown in colour. During the first precipitation the lignin loses 12–15 % of its weight. A second precipitation causes it to lose 2–3 %: in subsequent reprecipitation no measurable quantities of lignin are lost. After the second reprecipitation, the N, ash, and OCH_3 contents of the lignin remain constant. Lignin obtained in this manner still contains 1–2 % of ash.

The residue (1) remaining after solution of the lignin is again dissolved in warm 1·25 N-NaOH. The solution is filtered or centrifuged, acidified with 5 N-H_2SO_4, and mixed with 5 vol. of 96 % ethanol, whereupon precipitation of yellow flakes takes place and the lignin still present dissolves in the ethanol. Solution and reprecipitation are repeated, until the supernatant fluid is colourless. The ethanolic solutions are collected and treated for a further yield of lignin. The precipitated flakes are suspended in water and washed by decantation till the complete disappearance of SO_4^{--}. The lignin fraction obtained by separation from the

Table 1. *Yields of lignin and nitrogen contents of the different lignins and the original materials*

Material		Yields from dried original material (%)	N contents: Lignin (%)	N contents: Dried original material (%)
Eragrostis tef	Plant	5·4	1·18	1·86
	Faeces	9·0	1·31	1·15
Pencillaria	Plant	4·6	1·21	1·68
	Faeces	7·1	1·40	1·68
Setaria italica	Plant	5·1	1·41	1·08
	Faeces	6·8	1·62	1·20
Wild barley	Plant	5·1	1·63	0·52
	Faeces	6·5	1·70	0·71
Bersem clover	Plant	5·0	2·92	2·99
	Faeces	4·6	3·17	2·16
Fahli clover	Plant	3·0	3·36	1·67
	Faeces	3·4	3·92	1·52
Peanut hay	Plant	5·2	3·24	3·12
	Faeces	6·3	3·45	3·28
Cyprus vetch	Plant	4·1	3·30	2·48
	Faeces	6·5	3·41	1·94

hemicellulose residue was always identical with the principal product (containing the same percentage of N and OCH_3) and, therefore, it is combined with the latter.

The combined precipitates of hemicelluloses are then suspended in acetone, in which they are boiled three times, filtered and dried at 40° *in vacuo*. This material, on hydrolysis with boiling 0·4N-H_2SO_4 left a residue of about 10% consisting of lignin. The rest was split completely into reducing sugars, and is therefore assumed to be a hemicellulose.

Yield and N content of the lignins prepared from different plant and faeces materials are presented in Table 1, and their OCH_3 contents in Table 3. In several cases the lignins obtained in the first to fourth extraction were precipitated and treated separately. The N and OCH_3 contents of the lignins prepared from the different extracts were practically uniform, thus eliminating the need for their separate treatment throughout.

Properties of lignin

The various lignins prepared by extraction with 0·5N-NaOH are light to dark-brown, fine powders, decomposing without melting on being heated to 400°. They are easily soluble in dilute NaOH, in ethanol, and in acetone. Up to 70% of the material is soluble in 98% acetic acid. It should, however, be noted that completely dry, glacial acetic acid will not dissolve lignin either cold or hot; solution only occurs after the addition of 2–3% of water. Phenol and β-naphthol dissolve up to 15% of their weight of lignin when heated about 20° above their melting points. Lignins are not soluble in other common solvents.

The lignins prepared by us proved to be completely free from carbohydrates. The hydrolysates obtained according to Waksman's method for the determination of hemicellulose and cellulose were always found to be completely free of reducing sugars (cf. Bondi & Meyer, 1943). Nor could furfural be obtained by distillation with 3N-HCl, as in the Tollens pentosan determination.

Nitrogen content of lignin

Wood lignin is practically free from N. Very small quantities of N (0·2–0·3%), found in wood lignin, are considered by Kalb (1923) and Paloheimo (1925) to be accidental impurities caused by protein materials hydrolyzable only with difficulty; and Waksman (1938) assumes that there are lignin proteinates present in humus lignin which are difficult to split. Phillips, Goss, Davis & Stevens (1939) found some N in crude lignin from oats, which decreased in quantity with increasing age of the plant. Our experiments show with certainty that the N content of lignin prepared from green plants is due neither to accidental contamination with protein nor to the presence of lignin-protein complexes. The constancy of the N content of lignin prepared from the same plant material in consecutive extractions and by different methods shows that there can be no question of fortuitous contaminations.

In order to avoid the formation of lignin-protein complexes during extraction, an attempt was made to remove the protein before extraction by treatment of the plant material with 0·05N-NaOH. It was found that 80–90% of the protein present in the original material was removed by this preliminary treatment, but that at the same time most of the lignin also went into solution. Lignins prepared from material that had undergone this preliminary treatment were practically equal in N content to those obtained without preliminary treatment. On the other hand, the yield of lignin was greatly reduced, so that this preliminary treatment with NaOH was of no practical use.

A trial was also made of treatment of the plant material with a solution of pepsin in dilute HCl in order to secure a diminution of the protein content. By this method, too, 60–75% of the protein present was removed; but the lignin from plants given this preliminary treatment showed approximately the same N content as that from plant material which did not receive it. This equal N content of lignins prepared from protein-rich plants and of those from which most of their protein had been removed, argues against the hypothesis that condensation between lignin and protein takes place during the extraction of lignin.

Finally, experiments were undertaken which were intended to find out whether the N in lignin was protein N or not. The N content of the lignins was not diminished by vigorous acid hydrolysis (refluxing for 24 hr. with 5N-H_2SO_4) or by mild hydrolysis (digestion with 0·5N-HCl for 24 hr. at 38°). Both treatments bring about the dissolution of 10–15% of the lignin, but the solutions do not contain N and the N content of the remaining lignin rises proportionally to the weight loss. Repetition of the hydrolytic treatment leaves the lignin completely unchanged. The same result was obtained when pepsin was added to the HCl solution. The N-containing portion of the lignin was equally resistant to pancreatin at its optimum pH of 8·0. At this pH lignin is soluble, but no degradation of the lignin N could be obtained by this method. The resistance of the lignin N to hydrolysis by mineral acids or by enzymes shows that it cannot be protein N. Additional evidence for the non-protein nature of lignin N is furnished by the fact that the N content of faecal lignin is not lower than that of plant lignin, i.e. the digestive enzymes of the ruminant have no influence whatsoever on lignin N.

Nor can lignin N be primary or secondary amine N, as is shown by the resistance of lignin N to distillation of the lignins with concentrated NaOH and to treatment of the lignins with $NaNO_2$ (no liberation

of N occurred and the N content of the lignin remained unchanged). Therefore, the only possibility left is that the N is present as tertiary N, possibly as a cyclic compound. In this connexion it may be mentioned that derivatives of pyridine have been found in humus lignin (see Waksman, 1938), and that pyridine and its homologues appear regularly in the dry distillation of wood.

In order to test this assumption of the presence of heterocyclic N compounds in lignin, the following experiments were carried out.

According to Shaw (1937) pyridine decomposes on reduction with Na in absolute ethanol to give NH_3 and glutardialdehyde. Our lignins were, therefore, also subjected to reduction with Na and ethanol, as well as with Zn dust in alkaline solution. These treatments degraded the lignin completely to N-free products of low molecular weight, while all the N is driven off as NH_3 during the reaction with Zn dust, and may be collected in acid and determined by back-titration of the latter. (In one set of experiments, 50–100 hr. were necessary for complete liberation of the lignin N, when 5·0 g. lignin were refluxed with 60 g. Zn dust, 100 g. NaOH and 450 ml. water.)

Similar results were obtained by reducing the lignin with Na in ethanol: 10 g. lignin were refluxed in 300 ml. absolute ethanol, while 50 g. Na were added in small portions. The reaction was terminated in 30–40 hr., lignin N being recovered as NH_3.

Preliminary experiments showed that oxidation of lignin with $KMnO_4$ in alkaline solution also produced NH_3. These experiments were then repeated quantitatively, in accordance with the method used by Charnbury, Eckert, LaTorre & Kinney (1945) in investigating the nature of the N in humic acids. This determination showed that all lignins yield 70–80 % of their N content as NH_3 in the course of oxidation. For comparison, a derivative of pyridine, nicotinic acid, was subjected by us to the same treatment and gave similar results (see Table 2). The similar behaviour of the N contained in nicotinic acid and in our lignins allows us to infer that the N in lignins and in compounds containing tertiary N is bound similarly.

Table 2. *Liberation of nitrogen of lignins as ammonia by oxidation with* $KMnO_4$

Lignin from		Percentage N before oxidation	Loss of N as percentage of total N
Eragrostis tef	Plant	1·18	76·2
	Faeces	1·31	75·0
Pencillaria	Plant	1·21	73·9
	Faeces	1·41	76·2
Wild barley	Plant	1·63	77·8
	Faeces	1·70	78·1
Fahli clover	Plant	3·36	74·6
	Faeces	3·91	75·9
Nicotinic acid		9·44	78·4

Hydroxyl and methoxyl groups in lignin

The OCH_3 content of different wood lignins varies from 14 to 20 %, and is higher in hardwood lignins than in softwood lignins. According to Phillips *et al.* (1939) and Bondi & Meyer (1943) the OCH_3 content of lignin isolated from green plants is lower than that of wood lignin, and increases with the age of the plant. In barley, for instance, the OCH_3 content of the lignin increases during growth from 3 to 10 % (Phillips *et al.* 1939).

Table 3 gives the OCH_3 contents of the various lignins isolated by us from plants and faeces. These figures fall naturally into two groups: lignin from the Gramineae contains 9–10 % OCH_3, while the lignin of the Leguminosae contains approximately

Table 3. *Methoxyl content of lignins*

Lignin from		Percentage OCH_3 in lignin		
			After methylation	
		As prepared	With CH_2N_2	With $(CH_3)_2SO_4$
Eragrostis tef	Plant	9·71	19·00	23·48
	Faeces	9·70	19·02	19·34
Pencillaria	Plant	9·65	18·77	22·75
	Faeces	9·65	19·03	19·10
Setaria italica	Plant	9·60	18·94	23·04
	Faeces	9·70	18·81	18·92
Wild barley	Plant	9·85	19·21	21·33
	Faeces	9·77	18·48	18·59
Bersem clover	Plant	5·10	15·29	18·08
	Faeces	5·22	15·01	15·10
Fahli clover	Plant	5·75	15·31	16·70
	Faeces	6·67	15·42	15·51
Peanut hay	Plant	4·28	13·4[illegible]	15·20
	Faeces	4·40	14·[illegible]	14·09

one half of this amount. It is notable that a material like peanut hay, which on inspection appears much more woody than, say, barley, nevertheless shows clearly by the OCH_3 content of its lignin that it belongs to the Leguminosae; this shows that the family to which the plant belongs has, at least in this case, a stronger effect than the age and degree of 'woodiness'.

Table 3 also shows that the OCH_3 content of the lignin isolated from faeces differs hardly at all from that of the corresponding plant lignins; i.e. there is no noteworthy rupture of ether linkages in the alimentary tract.

The determination of OCH_3 in lignin was supplemented by the estimation of OCH_3 introduced into the lignin. All samples of lignin were methylated by two different methods, with dimethyl sulphate and with diazomethane, according to the procedures given by Phillips (1944).

The results of OCH_3 determinations in the methylated lignins are also given in Table 3. The OCH_3 content of lignins from Leguminosae is trebled on methylation with diazomethane, whilst the content of Gramineae lignins is only approximately doubled. We conclude from this that the OCH_3 groups in lignins from Gramineae (which have the higher OCH_3 content before methylation) correspond to unsubstituted hydroxyl groups in the lignins from Leguminosae. Methylation with dimethyl sulphate brings about only slightly larger increases in OCH_3 content than that obtained with diazomethane. Comparison of the results of methylation with dimethyl sulphate and diazomethane therefore shows that in all plant lignins there are many more phenolic (and enolic) hydroxyl groups than aliphatic ones. Since faeces lignins, unlike plant lignins, gain the same amount of OCH_3 by methylation with dimethyl sulphate and with diazomethane, it seems that in faecal lignins non-phenolic hydroxyl groups are completely absent.

Determination of the molecular weight of lignin

The values in the literature of the molecular weight of lignin vary widely. In earlier publications Beckman & Liesche (1921*a*, *b*) found, by cryoscopic determinations in phenol, values of 762 and 784 for the molecular weight of lignin prepared from wheat straw; later Fuchs (1936) found a value of 900. More recently, Conner (1941) determined a value of 3900 by dielectric measurements. These figures are comparatively low values for high polymers, but become more plausible when we consider that the properties of lignin are different from those of other high-polymer plant materials. The structure of lignin is not fibrous and the viscosity of its solution is low.

Freudenberg (1939) assumes that lignin has a three-dimensional structure created by the polymerization of from 2 to 10 molecules of coniferyl alcohol. This would indicate that the molecular weight was comparatively low. Substances like magnalol and lariciresinol (the so-called lignanes, containing 2 or 3 hydroxyconiferyl alcohol molecules) have an even lower molecular weight (see Haworth, 1942).

The differences between the molecular weight obtained by the cryoscopic method and those obtained by other physical methods appear to be due to the depolymerization of the lignin by the solvents used in the cryoscopic determination, so that the result is the molecular weight, not of the lignin, but of the 'structural units' of which it is composed. Furthermore, the molecular weights given in Table 4 should be regarded as averages only, since there is no evidence that the lignin molecule breaks down to give units identical in molecular weight. The molecular weights given in Table 4 should be accepted with this reservation. It should be noted that the molecular weights of lignins obtained from plants and from faeces are of the same order of magnitude.

The lignins used for cryoscopic determination of their molecular weight were purified by twice dissolving in 98 % acetic acid, reprecipitating by dilution with ice water and by drying in a vacuum over solid NaOH at room temperature. β-Naphthol was employed as a solvent, its molecular freezing point depression was determined by Bruni (1923) as 11·25°. The determinations were carried out in a conventional Beckmann micro-apparatus with an electromagnetic stirring device; glycerol was used in the bath for maintaining temperatures between 115 and 130°.

Table 4. *Molecular weight of lignins determined by depression of freezing point of β-naphthol*

Lignin from		Weight of lignin (g.)	Weight of β-naphthol (g.)	Δt	Molecular weight
Pencillaria	Plant	0·1034	2·1272	0·861	635
	Faeces	0·1078	2·2964	0·804	657
Wild barley	Plant	0·1661	1·9470	1·500	640
	Faeces	0·1388	1·8696	1·327	629
Fahli clover	Plant	0·2076	1·7250	2·205	614
	Faeces	0·1240	2·2095	1·042	606
Peanut hay	Plant	0·1002	0·8228	2·203	621
	Faeces	0·1228	1·5180	1·497	608

Degradation of the lignins

A number of degradation experiments were undertaken with the purpose of comparing the degradation products obtained from plant and faeces lignins and from wood lignins. The processes used in this investigation were the following: (1) oxidation with nitrobenzene in alkaline solution; (2) fusion with potassium hydroxide; (3) hydrolysis with acid ethanol under pressure.

(1) *Oxidation of lignins with nitrobenzene and alkali.* This reaction, which was successfully used by Freudenberg, Lautsch & Engler (1940) and by Creighton, Gibbs & Hibbert (1944), consists in treatment in NaOH solution with nitrobenzene at 160–200° under pressure for several hours. After removing the reduction products of the nitrobenzene by steam distillation the solution is acidified and extracted with ether. The ether solution is successively extracted with solutions of $NaHSO_3$, $NaHCO_3$ and NaOH, to separate aldehydes, acids and phenols, respectively. Four 30 g. samples of each lignin were treated with nitrobenzene and were combined before the ether extraction. The aldehyde extracts were acidified and SO_2 removed *in vacuo*, after which the solution was divided into two parts. The total aldehydes were precipitated from one part with a 2% solution of 2:4-dinitrophenylhydrazine in 2N-HCl. The precipitate was filtered off, washed with water, dried and the OCH_3 content was determined. From the other part of the aldehyde fraction the aldehydes were extracted with ether; this was removed and the aldehydes sublimed in a high vacuum.

A particularly remarkable fact is the total absence from the plant lignins investigated by us of syringaldehyde, a characteristic component of the aldehyde fraction of hardwood lignins. When the aldehydes were distilled at a pressure of 0·1 mm. no fraction could be obtained with a higher b.p. than 150° (syringaldehyde distils at 220–250° at this pressure). In the distillate, vanillin and *p*-hydroxybenzaldehyde could be separated, according to Creighton & Hibbert (1944), by means of their different solubilities in benzene and identified by mixed melting points. The proportion of the two aldehydes could be calculated in each case from the OCH_3 content of the 2:4-dinitrophenylhydrazones.

The total yields of aldehydes and the proportions of vanillin and *p*-hydroxybenzaldehyde are given in Table 5. It may be mentioned for comparison that Creighton *et al.* (1944) isolated 35–51% of aldehydes (syringaldehyde and vanillin) from hardwood lignins and 15–24% of vanillin from softwood lignins. *p*-Hydroxybenzaldehyde is not present in the aldehyde fraction of wood lignins and only 1·8% was obtained by Creighton & Hibbert (1944) from maize-cob lignin.

Table 5 shows that the quantity of aldehydes obtained from the oxidation of plant lignins with nitrobenzene varies within wide limits, while the aldehyde fraction is almost completely absent from the nitrobenzene oxidation product of faecal lignins. It is remarkable that Gramineae lignins give a far larger quantity of aldehydes on oxidation than lignins from Leguminosae. Here, too, the botanical family to which the plant belongs has a far greater influence on the composition of the lignin than the degree of 'woodiness', as may be seen in the case of peanut hay, which belongs to the Leguminosae. Though the total percentage of aldehyde obtained varies greatly, the ratio of vanillin to *p*-hydroxybenzaldehyde remains constant throughout and is approximately 2 : 1, without being influenced either by the botanical family or the degree of woodiness of the plant from which the lignin is derived.

The absence of the aldehyde fraction from the oxidation products of faecal lignins shows that in the body the lignin undergoes a change, which renders it incapable of yielding aldehydes on degradation with nitrobenzene.

Table 5. *Formation of aldehydes by oxidation of lignins and nitrobenzene*

Lignin from		Aldehydes (as percentage of the lignin)	Vanillin (as percentage of total aldehydes)	*p*-Hydroxybenzaldehyde (as percentage of total aldehydes)
Eragrostis tef	Plant	19·2	64·3	35·7
	Faeces	0	0	0
Pencillaria	Plant	16·3	63·8	36·2
	Faeces	0·31	0	—
Setaria italica	Plant	16·1	66·1	33·9
	Faeces	0·26	—	—
Wild barley	Plant	21·6	66·6	33·4
	Faeces	0·25	—	—
Bersem clover	Plant	4·2	65·4	34·6
	Faeces	—	—	—
Fahli clover	Plant	4·6	66·0	34·0
	Faeces	0·15	—	—
Peanut hay	Plant	8·3	64·7	35·3
	Faeces	0·10	—	—
Cyprus vetch	Plant	6·3	62·6	37·4
	Faeces	0·04	—	—

Benzoic acid and phenol were the main constituents of the acid and phenolic fractions, respectively; no importance can, however, be attributed to them, since they probably originated from the nitrobenzene, as was shown by oxidizing sugars instead of lignins by the same method, when no aldehydes, but considerable quantities of both benzoic acid and phenol, were formed.

(2) *Degradation of the lignins by alkali fusion.* The lignins were fused with KOH at 250–300° according to the method of Heuser & Winsvold (1923). A total of 100 g. of each lignin was treated in batches of 5 g. The melt was diluted with water, acidified and extracted with ether. After evaporation of the ether, catechol and protocatechuic acid could be separated in the residue by their different solubilities in benzene and identified by colour reactions (with $FeCl_3$ and $AgNO_3$), and by identification of their methylation products, veratrol and veratric acid (CH_3O contents and mixed melting points).

Lignins from Gramineae yield 1·36–2·25 % of protocatechuic acid and 0·20–0·40 % of catechol on alkali fusion. Lignins from Leguminosae yield 0·20–1·00 % protocatechuic acid and 0·30–0·56 % of catechol. In this case, no difference between plant and faecal lignins could be found.

(3) *Treatment with acid ethanol (ethanolysis).* By 'ethanolysis', i.e. by refluxing the lignins for 40–100 hr. with absolute ethanol containing 3 % by weight of HCl, Cramer, Hunter & Hibbert (1939) obtained a considerable number of well-defined degradation products of wood lignin. We attempted to use this method for the investigation of plant lignins, but obtained only small amounts of degradation products, most of the lignin being converted into a black carbonized mass. For this reason we modified this method by using aqueous ethanol instead of absolute, and by working under pressure, which permitted work at a higher temperature and, therefore, a very considerable reduction of the reaction time.

The detailed procedure was as follows: 10 g. of lignin were dissolved in 40 ml. of 2·5 N-NaOH with gentle heating, and this solution was mixed with 350 ml. of 85 % (v/v) ethanol to which 10 ml. of conc. H_2SO_4 had been added. Ten batches of each lignin were treated in this manner. The solution of the lignin in NaOH and its reprecipitation with acid yielded a finely divided, reactive product. This mixture was now put in an autoclave with glass lining, and in the course of 2 hr. heated to 180–190°, at which temperature it was kept for 6–7 hr. The black solid residue was filtered off, dried, extracted with ether, and again subjected to the same treatment in 10 g. batches with acidified ethanol. After the ethanolysis had been repeated three times, there were usually only small quantities of unreacted lignin left. All solutions were now combined and 0·25 vol. of water was added. After evaporation of the ethanol *in vacuo*, the residue was extracted with ether, and the ether extracted successively with $NaHSO_3$, $NaHCO_3$ and NaOH solutions, and thus divided into aldehyde, phenol, acid and neutral fractions.

The yields of the various fractions are given in Table 6. As in the case of nitrobenzene oxidation, treatment by acidified ethanol yields much larger quantities of carbonyl compounds from Gramineae than from Leguminosae lignins. In both cases, practically no carbonyl compounds were formed from faecal lignins. The composition of the aldehyde fraction obtained by ethanolysis is, however, completely different from that obtained by nitrobenzene oxidation. The main part of this fraction was a light yellow oil which, after distillation under 0·05 mm., partially crystallized on cooling. It was assumed that this was a benzene derivative containing a side chain with an aldehyde or ketone group in it; many such compounds have been found by Hibbert (1942) and Freudenberg (1939). By addition of a Ni salt and hydroxylamine a brown complex Ni salt could be prepared, whose Ni content (determined by dimethylglyoxime after treatment with H_2SO_4) agreed with that calculated for 1-(4′-hydroxy-3′-methoxyphenyl)propane-1:2-dione dioxime (11·63 % Ni). This Ni salt was prepared according to Kulka, Hawkins & Hibbert (1941). 1-(4′-Hydroxy-3′-methoxyphenyl)propane-1:2-dione is the only material obtained in this investigation with a side chain containing three carbon atoms.

The phenol fraction was separated by fractional distillation into two portions, b.p. 180–190° and 200–210°. After redistillation of each fraction, phenol and guaiacol were identified as their principal components by colour reactions (with $FeCl_3$, and with $FeCl_3$ and $AgNO_3$, respectively) and by the mixed melting point of their 3:5-dinitrobenzoates.

Table 6. *Amounts of degradation products obtained by ethanolysis of lignins*

(As percentage of the starting material.)

Lignin from		Aldehydes	Phenol	Acids and neutral fraction	Total yield
Eragrostis tef	Plant	2·31	6·40	4·28	12·99
	Faeces	0·22	5·77	5·22	11·20
Setaria italica	Plant	1·98	6·24	2·32	10·54
	Faeces	0·22	4·00	3·45	7·67
Wild barley	Plant	2·56	7·83	3·22	13·61
	Faeces	0·18	2·20	3·18	5·56
Bersem clover	Plant	0·61	1·28	0·42	2·31
	Faeces	—	0·96	1·75	2·71
Fahli clover	Plant	0·84	2·65	0·33	3·82
	Faeces	0·04	1·21	2·02	3·27
Peanut hay	Plant	0·98	4·50	2·07	6·55
	Faeces	0·26	1·80	3·74	5·80

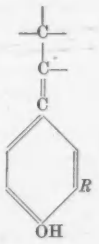
Vanillic acid was found along with *p*-hydroxybenzoic acid, in the combined acid and 'neutral' fractions (the latter being the compounds remaining after aldehydes, acids and phenols were removed). The 'neutral' and acid fractions were worked up together, because the acid fraction was always obtained in very small quantities only. The two fractions were subjected to oxidation with $KMnO_4$ by the following method:

The residue remaining after evaporation of the ether was dissolved in a little ethanol, 50 ml. of water were added and the mixture heated on the water bath. An excess of 4% $KMnO_4$ solution was then added. The mixture was heated on the steam bath for 1 hr. and filtered. The filtrate was acidified with H_2SO_4 and extracted with ether. The ether was evaporated and the residue distilled under 0·05 mm. Two fractions were obtained. One had a very low OCH_3 content (0·2%), and could be purified by digestion with ice-cold benzene and a second sublimation under high vacuum. Its identity with *p*-hydroxybenzoic acid was proved by the m.p. and mixed m.p., 213–215°, with an authentic specimen, m.p. 215°. The fraction obtained at higher temperature could be recrystallized from water and was afterwards re-sublimed in a high vacuum. Its m.p., 205–206°, and OCH_3 content proved its identity as vanillic acid.

DISCUSSION

The structure of the lignins

The degradation products obtained by us correspond to those obtained from wood lignins. It seems, therefore, that the unit:



(R = H or OCH_3)

assumed by Klason (1936) and by Hibbert (1942) and Freudenberg (1939) to be present in wood lignins is also an essential constituent of the lignins in young plants (see also Percival, 1942). The occurrence of vanillin and *p*-hydroxybenzaldehyde can easily be explained by the oxidative fission of the side-chain double bond. From model experiments carried out recently by Wacek & Kratzl (1947) it is apparent that only those aromatic compounds which contain an olefinic double bond between carbon atoms 1 and 2 in the side chain, e.g. 4-hydroxy-3-methoxycinnamic acid, give, on oxidation with nitrobenzene, vanillin in good yield, while compounds like 3:4-dimethoxybenzylacetone do not. The assumption that the aldehydes formed by nitrobenzene oxidation are derived from compounds containing a three-carbon side chain is supported by the finding of 1-(4′-hydroxy-3′-methoxyphenyl)propane-1:2-dione. However, the side chain is not split by ethanolysis and, therefore, no aldehydes can be found. Vanillic, *p*-hydroxybenzoic and protocatechuic acids result from a complete oxidation of the side chain to COOH, which is followed in some cases by the loss of CO_2, thus leading to the formation of catechol, guaiacol and phenol. The constant proportion of vanillin to *p*-hydroxybenzaldehyde (2 : 1) observed by us seems to point to the fact that a lignin structural unit contains three aromatic nuclei, two yielding vanillin on oxidation and one *p*-hydroxybenzaldehyde (the loss of a OCH_3 group by oxidation with nitrobenzene has never been observed and seems improbable).

As already pointed out on p. 253, wood lignins yield, on oxidation with nitrobenzene, considerable quantities of aldehydes rich in OCH_3, a fact which is in accordance with the larger OCH_3 contents of wood lignins in general.

The OCH_3 content of the Gramineae lignins shows that each structural unit (assuming its molecular weight to be 625) must contain two OCH_3 groups (theory, 9·9% OCH_3); see Table 3. Methylation with diazomethane raises the OCH_3 content to 19%, which shows that two phenolic OH groups are methylated. Since the experimental evidence points to the presence of three aromatic rings, two of them substituted with OCH_3 and OH and the third with OH only, one must necessarily assume that the remaining phenolic OH group is attached in such a position as to make it incapable of being methylated with diazomethane.

Leguminosae lignins contain only one OCH_3 group per structural unit (theory, 5·1%) and show on methylation with diazomethane an increase in OCH_3 content which corresponds to the presence of two phenolic OH groups. Since, on oxidation with nitrobenzene of the Leguminosae lignins, vanillin and *p*-hydroxybenzaldehyde are also formed in the proportion 2 : 1 (although in smaller yields), one must assume that part of the units still contains a system of two rings, each of them substituted with OH and OCH_3, and one ring substituted with OH only. This would mean that half of the Leguminosae lignin is methylated in the usual manner, i.e. it contains two OCH_3 groups in one structural unit. The other half is not methylated at all. A large part of the free phenolic OH groups in Leguminosae lignins is apparently incapable of being methylated.

Plant lignins take up one OCH_3 group more on methylation with dimethyl sulphate than on methylation with diazomethane indicating the presence of one non-phenolic OH group.

Plant lignins contain, in contrast to wood lignins, nitrogen as a characteristic component. All lignins

can be classified by their increasing N and decreasing OCH_3 content as follows:

Hardwood lignins, N, nil; OCH_3, 20%.
Softwood lignins, N, 0·2–0·3%; OCH_3, 15%.
Gramineae lignins, N, 1·2–1·6%; OCH_3, 10%.
Leguminosae lignins, N, 2·9–3·4%; OCH_3, 5%.

Digestion of lignins

The solubility of the lignins, the apparent molecular weight of the building units, and the OCH_3 and N content remain unchanged by digestion. On the other hand, the disappearance of the aldehyde fraction from among the degradation products of faecal lignins shows that changes in the side chains of the aromatic rings have occurred, probably by hydrogenation of the side-chain double bond, preventing their oxidative fission and the formation of aldehyde groups. The other significant change is the complete disappearance of the non-phenolic OH group indicated by the fact that the increase in OCH_3 content, obtained by methylation with dimethyl sulphate, is identical with that obtained with diazomethane.

SUMMARY

1. Lignins were prepared by extraction with dilute alkali from a number of annual forage plants and from faeces collected from sheep fed exclusively with the same plants. The plants used were *Eragrostis tef*, *Pencillaria*, *Setaria italica*, *Hordeum murinum* (wild barley), *Trifolium alexandrinum bersem*, *T. alexandrinum fahli*, *Lathyrus ochrus* and hay of *Arachis hypogea* (peanut).

2. All lignins contained nitrogen whose presence is not due to accidental contamination with proteins, but seems to be a characteristic component of the lignin molecule itself. The methoxyl content of the lignins ranges from about 5% in Leguminosae lignins to about 10% in Gramineae lignins.

3. On methylation with diazomethane, the original methoxyl content of the Leguminosae lignins is trebled, that of the Gramineae lignins doubled. Methylation with dimethyl sulphate brings about a small increase in methoxyl uptake over that obtained with diazomethane. In faecal lignins no additional methylation with dimethyl sulphate could be obtained.

4. Cryoscopic molecular weight determinations in β-naphthol showed values of about 600 both for plant and faecal lignins.

5. Oxidation of the plant lignins with nitrobenzene in alkali yielded vanillin and *p*-hydroxybenzaldehyde. Faecal lignins did not yield any aldehydes. The ratio of vanillin to *p*-hydroxybenzaldehyde was always 2 : 1.

6. On fusion with potassium hydroxide all lignins gave catechol and protocatechuic acid.

7. On heating the lignins with ethanol containing 3% sulphuric acid to 170–180° under pressure, 1-(4′-hydroxy-3′-methoxyphenyl)propane-1:2-dione was obtained only from plant lignins; both plant and faecal lignins yielded phenol, guaiacol and an acid fraction, which, after further oxidation with $KMnO_4$, yielded vanillic and *p*-hydroxybenzoic acids.

8. The experimental results are discussed in relation to the structure of the lignins.

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Penicillin: Analysis of the Crude Product by Means of a Modified Partition Chromatogram

1. THEORY AND USE OF THE CHROMATOGRAM

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Crude penicillin isolated from solvent extracts of metabolism solutions (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941; Abraham, Chain & Holiday, 1942), as a calcium, barium or sodium salt, is a yellow to brown solid which may contain well over thirty different acidic substances, mostly coloured. Still further weakly acidic and neutral substances remain in the residual solvent phase. The physical and chemical properties of the substances are such that chromatography provides the only likely method for the separation of the components of the mixture. So far, however, few adsorbents have been found which give satisfactory chromatograms. A modified partition chromatogram has been described (Catch, Cook & Heilbron, 1942) based on that of Martin & Synge (1941), but this chromatogram is capable of little development although considerable purification can be achieved.

The present paper describes work begun in 1942 upon a modification of the Martin & Synge (1941) partition chromatogram, which has been found suitable for substances of the nature of the acids in crude penicillin, and which can be developed. This chromatogram consists of silica gel impregnated with strong solutions of potassium phosphate of a suitable acidity. With this technique it has been possible to demonstrate in crude penicillin the existence of at least five chemical individuals with varying degrees of antibacterial power, although complete purification of any one of them by this method alone has not been achieved.

In the earlier stages of this work the position of the active zones among the numerous visible coloured bands could only be located by biological activity, the test results for which were not available until 24 hr. after the column had been run. Partition coefficient determinations in tap funnels under conditions comparable to those on the columns were, therefore, carried out and the results applied to the columns according to the theoretical treatment of Martin & Synge (1941). This proved a valuable guide to the approximate position of the main active zone, but as larger columns and heavier loads were used it became apparent that this treatment was inadequate, as indeed its authors imply (Martin & Synge, 1941). By this time experience enabled us to locate the desired bands with some certainty from the appearance of the column, and from the position of a prominent orange band associated with the colour reaction described by Catch *et al.* (1942). On our column this band was substantially inactive and occurred a little below the main active zone. Still later the polarimeter was of great assistance because the biological activity was always associated with a strong dextrorotation, other constituents being usually laevorotatory or optically inactive.

It was clearly desirable to define as closely as possible the best conditions for carrying out various types of separations, since it was obvious that there was much overlapping of bands. In particular the separations of penicillins I, II and IV proved to be very difficult (cf. Part 2, Boon, Calam, Carrington & Freeman, 1944; Boon, Carrington & Levi, 1944). An attempt was made, therefore, to apply the recent theories of de Vault (1943) and Weiss (1943) to this type of column. A practical test of the theory was then made with phenylacetic acid and benzoic acid as model substances.

THEORETICAL TREATMENT

Consider a column prepared as described below of length L cm., and cross-sectional area A sq.cm. containing M g. of silica gel (sp.gr. D) impregnated with K ml. of a buffer solution. The column is initially filled with solvent:

Area of silica phase $= M/LD = I$.

Area of buffer phase numerically equal to volume of buffer/cm. of column $= K/L = P$.

Area of solvent phase numerically equal to pore volume/cm. of column $= A - I - P = S$ (Martin & Synge, 1941).

Consider a single solute added to the column in a volume of solvent v_0, so that its concentration is C_0.

Let V be the total volume of filtrate at any time, and v the volume of filtrate after the commencement of development where $v = V - v_0$.

Let x be the distance of any point from the top of the column.

Let C be the concentration of the solute at any point in the solvent phase, and $B\ (=f(C))$ be the concentration in the buffer phase.

For the partition chromatogram the differential equation deduced by de Vault (1943) becomes

$$\frac{\partial C}{\partial x}+\frac{\partial C}{\partial v}[S+Pf'(C)]=0, \quad \text{where } f'(C)=\frac{dB}{dC} \qquad (1)$$

or

$$x=F(C)+\frac{v}{S+Pf'(C)}, \qquad (2)$$

where $F(C)$ is an arbitrary function to be defined by the initial conditions.

From this equation and a detailed consideration of the processes on the column analogous to that of de Vault (1943) and Weiss (1943) we obtain the following relations.

Initial width of band (x_1). This can be found from equation (2), or more simply as follows.

Of the total quantity of solute v_0C_0 the amount in the mobile phase is x_1SC_0, and the amount in the stationary buffer phase is $x_1Pf(C_0)$.

Hence

$$v_0C_0=x_1SC_0+x_1Pf(C_0)$$

or

$$x_1=\frac{v_0C_0}{SC_0+Pf(C_0)}=\frac{v_0}{S+P(B_0/C_0)}. \qquad (3)$$

Lower edge of constant concentration zone (x_3). Since the concentration in the solvent phase at the front of the band is still C_0 the lower edge of the zone moves as though the original solution were still being poured into the column. Hence we can write, as for x_1,

$$x_3=\frac{VC_0}{SC_0+Pf(C_0)}=\frac{V}{S+P(B_0/C_0)}. \qquad (4)$$

Upper edge of constant concentration zone (x_2). For the point x_2 if volumes are measured from the commencement of development $F(C)=0$ (de Vault, 1943) and equation (2) becomes

$$x_2=\frac{v}{S+Pf'(C_0)}. \qquad (5)$$

Concentration in the 'tail'. Concentrations can only be conveniently measured as the band leaves the column, i.e. when $x=L$. Measuring v from the commencement of development as before $F(C)=0$, and

$$L=\frac{v}{S+Pf'(C)} \quad \text{or} \quad f'(C)=\frac{v}{LP}-\frac{S}{P}. \qquad (6)$$

From this expression concentrations at any point in the 'tail', as it emerges from the column can be determined.

Volume (v_m) *at which the constant concentration zone just disappears.* When this occurs $x_2=x_3$. On equating the expressions for these we obtain

$$v_m=\frac{v_0C_0\{S+Pf'(C_0)\}}{P\{f(C_0)-C_0f'(C_0)\}}. \qquad (7)$$

It is seen that the theory of Martin & Synge gives an equation of the same form as (3) and (4) above, the only difference being the substitution of partition ratios for partition coefficients.

EXPERIMENTAL

Distribution experiments with benzoic and phenylacetic acids

Partition coefficients of phenylacetic acid at different acidities using concentrated buffer solutions. A stock solution of phenylacetic acid (2·72 g.; 20 m-equiv.) in damp diethyl ether (100 ml.) was prepared. Of the solution 10 ml. required 18·5 ml. of approximately 0·1 N-NaOH. A series of buffer solutions covering the approximate range pH 4·5–7·5 was prepared by mixing suitable volumes of 25% (w/w) KOH and H_3PO_4 until the desired pH was obtained after dilution 1 in 4. The solutions were saturated with ether before use. Quantities of the stock solution (2 ml.) were diluted to 20 ml. with ether, and each was shaken with 20 ml. of one of the buffer solutions. The pH of the buffer phase was measured after the partition. Sample parts of the ether phase were titrated. The concentration of acid in the buffer phase was estimated by difference. The results are shown in Fig. 1.

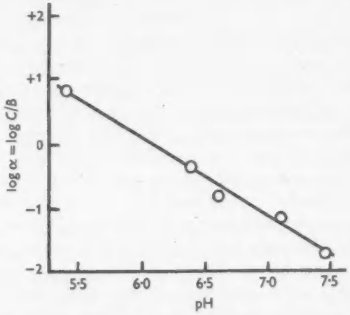

Fig. 1. Relation between partition ratio and pH for phenylacetic acid in ether and strong buffer solutions.

Effect of concentration on distribution between the phases. (*a*) *Phenylacetic acid.* The procedure was similar to that used in the previous experiment, with one buffer solution (pH 6·84) and solutions of phenylacetic acid in diethyl ether of varying concentration. Concentrations are conveniently expressed in m-equiv./l. The results are given in Table 1.

(*b*) *Benzoic acid.* The experiment was carried out in the same way as the one for phenylacetic acid using buffer solutions of pH 6·72. The results are given in Table 2.

Partition chromatogram with phenylacetic aci

Silica gel (Martin & Synge, 1941; Gordon, Martin & Synge, 1943) (90 g.; 90–160 mesh) was thoroughly mixed in a mortar with concentrated potassium phosphate buffer prepared as previously described (pH 6·36; 45 ml.) and the resulting, apparently dry, powder was suspended in ethylene dichloride (500 ml.) and poured into a chromatogram tube (3·68 cm. diam.). After the column had ceased to shrink (final dimensions 27 × 3·68 cm.) the ethylene dichloride was displaced with diethyl ether. Phenylacetic acid (2 g.) in diethyl ether (200 ml.) was then applied to the top of the column, and development was effected with moist ether; 200 ml. of filtrate were collected as a first fraction followed by further fractions of 20 ml. The concentration of phenylacetic acid in the eluate was determined by titration with standard $Ba(OH)_2$. For this column the following values were calculated by the method of Martin & Synge (1941):

Pore vol./cm. numerically equal to area of solvent phase (S) = 7·49 ml.

Vol. of buffer/cm. numerically equal to area of buffer phase (P) = 1·67 ml.

Table 1. *Effect of concentration on the distribution of phenylacetic acid between ether and concentrated buffer solutions*

(Acidity of buffer solutions was pH 6·82.)

Concentration in ether phase (m-equiv./l.)	log	Buffer phase (m-equiv./l. observed)	log	Buffer phase calculated from regression equation (m-equiv./l.)
2·66	0·4249	14·3	1·1553	15·2
5·8	0·7634	30·8	1·4886	27·6
15·2	1·1818	56·1	1·7490	57·4
15·3	1·1847	57·8	1·7619	57·7
19·7	1·2945	69·6	1·8426	70·0
44	1·6435	134	2·1271	128
104	2·0170	242	2·3838	245

Table 2. *Effect of concentration on the partition of benzoic acid between ether and concentrated buffer*

(Acidity of buffer solutions was pH 6·72.)

Concentration in ether phase (m-equiv./l.)	log C	Concentration in buffer phase (m-equiv./l.)	log B
27·6	1·44	69·3	1·84
59·8	1·78	123	2·09
168	2·23	239	2·38

Chromatograms with mixtures of benzoic and phenylacetic acids

Results of four experiments are illustrated in Fig. 2. The experiment illustrated in Fig. 2A was carried out with the same column as used for the above experiment with phenylacetic acid. The other three were carried out with a single new column differing only slightly in dimensions but with a buffer solution of higher pH (6·7).

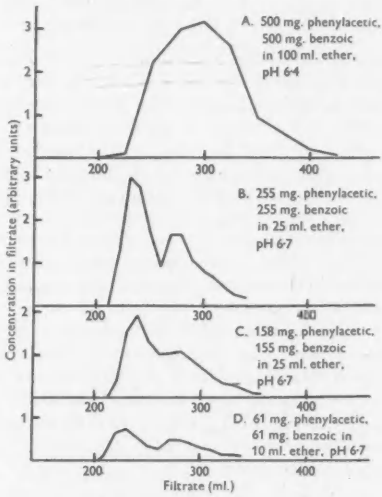
A. 500 mg. phenylacetic, 500 mg. benzoic in 100 ml. ether, pH 6·4
B. 255 mg. phenylacetic, 255 mg. benzoic in 25 ml. ether, pH 6·7
C. 158 mg. phenylacetic, 155 mg. benzoic in 25 ml. ether, pH 6·7
D. 61 mg. phenylacetic, 61 mg. benzoic in 10 ml. ether, pH 6·7
Concentration in filtrate (arbitrary units)
Filtrate (ml.)

Fig. 2. Mixtures of phenylacetic and benzoic acids; partition chromatogram.

DISCUSSION

In order to evaluate the expressions obtained in the theoretical portion it was first necessary to evaluate $f(C)$, i.e. to determine the effect of concentration on the partition ratio. For benzoic and phenylacetic acids the experimental results can be satisfactorily expressed in the form $f(C) = B = aC^b$ resembling the Freundlich adsorption isotherm where a and b are constants for the particular substance and pair of phases. It probably has little theoretical significance, and only holds for a comparatively narrow range of conditions since at low concentrations the value of B/C should approach a constant value whereas the above expression would make B/C continue to increase with decreasing C, i.e. b is not truly constant, but tends to unity as the concentration decreases. Nevertheless, over the range of concentrations normally employed in chromatography, the expression is sufficiently accurate for most purposes.

The calculated regression line for the results given in Table 1 is $\log B = 0{\cdot}86 + 0{\cdot}76 \log C$, i.e. $B = 7{\cdot}2C^{0{\cdot}76}$ at pH 6·84. At pH 7·4, $a = 16{\cdot}98$, $b = 0{\cdot}76$. The corresponding figures for benzoic acid at pH 6·72 are $a = 7{\cdot}24$, $b = 0{\cdot}68$. The value of b will probably normally lie well within the limits 0·5 and 1·0 (Note 1). The value of a is the value of B when $C = 1$, and approximates to the limiting partition ratio if suitable units are chosen.

The best fitting equation for the line shown in Fig. 1 is $\log (C/B) = 7{\cdot}32 - 1{\cdot}20\,\mathrm{pH}$. By means of this result and the value $b = 0{\cdot}76$ obtained above for phenylacetic acid it is possible to calculate $f(C)$ and $f'(C)$ for the chromatogram using phenylacetic acid. The appropriate expressions are $f(C) = 2{\cdot}82C^{0{\cdot}76}$;

$f'(C) = 2{\cdot}14C^{-0{\cdot}24}$, and from these the following may be calculated:

Position of front edge of band: 250 ml. (from equation (4)),

End of constant concentration band: 436 ml. (from equation (5)).

These are in reasonably good agreement with the experimental results, particularly at the front edge of the band, as the small overlap shown in Fig. 3

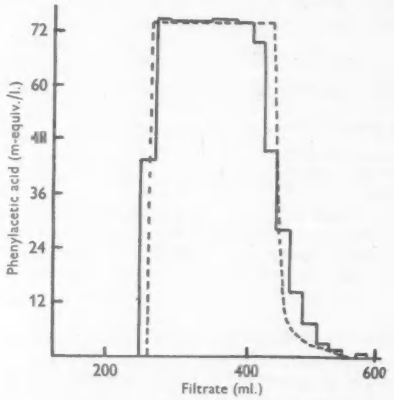

Fig. 3. Phenylacetic acid on a partition chromatogram. Full line, observed; dotted line, calculated.

is due to the fact that fractions were taken at 240 and 260 ml., and thus would include some eluate containing no acid. This sharpness of the front edge of the band indicates that other factors (diffusion, non-attainment of equilibrium, etc.) are relatively unimportant. The observed tail end of the constant concentration zone appeared a little earlier, and the fall off in the tail was slower than anticipated. It is thought that this is almost entirely due to the practical impossibility of achieving sharp boundary conditions when changing from solution to pure solvent. Thus, even if there is little mixing of the layers of liquid where they enter the column, some solute will be washed down from the walls of the tube above the column, especially with volatile solvents. In earlier experiments with this technique it was usual to add washings from the various vessels to the column, after the bulk of the charge had been added, for the sake of a good recovery. This was clearly a mistake and made the banding unnecessarily diffuse.

The results for the chromatograms of mixtures of benzoic and phenylacetic acids are of particular interest since the difficulty in separating acids was of about the same degree as the separation of penicillins I and II. In experiment A not only is the pH low, but the initial volume is too great so that the bands largely overlap. The higher pH and lower concentrations of experiments B, C and D proved much more effective (Fig. 2). These experiments were not carried further as ample examples of the practical application of the technique were obtained during the course of its use with crude penicillin (see Boon, Calam, Gudgeon & Levi, 1948).

It is probable that it will seldom be of value to apply the above equations in practice (especially since no account has been taken of the effect of one band on another) except in the case of colourless substances, or for the purposes of identification, and indeed our main interest in the theory was to define the best conditions for the treatment of the very complex mixture in crude penicillin. In this case, before the isolation of the pure substances, $f(C)$ cannot be determined so that a rigid application of the theory is impossible. Thus, as will be shown in later papers, many of the constituents occur close together on the column, and the theory was of assistance in defining conditions under which they could be separated.

Some of the practical consequences of the theory are set out below:

(1) On a given column the initial width of a band depends mainly on the total volume of the starting solution (equation (3)) relative to the size of the column. If the equation is written in the form

$$x_1 = \frac{v_0}{S + (PB_0/C_0)} = \frac{v_0}{S + PaC_0^{b-1}},$$

and taking the values quoted above for b, it is seen that for a given v_0 the band width will vary only slowly with change of concentration. The band width will be less at higher pH values (increasing a).

(2) As a corollary to this, to separate two substances whose partition relations lie close together on a given column, a small initial volume and a high pH will give the best conditions. It will be preferable that the constituent in greater amount should be the less soluble in the buffer phase, since the rate of movement of the band is increased by increased concentration.

(3) It is desirable that the material in a band should be mainly concentrated at the head of the band, and not too spread out into the 'tail', i.e. v_m in equation (7) should be a maximum. (It is not, however, essential that v_m should not be exceeded.) Substituting the values for $f(C)$ and $f'(C)$ in equation (7) we have:

$$v_m = \frac{v_0 C_0 S}{PB_0(1-b)} + \frac{v_0 b}{(1-b)}.$$

This expression is a maximum when v_0 is large, and when the pH is low. These conditions are the opposite of those in (2) above, so that in practice compromise conditions must be sought. Prolonged

development will give a wider separation at the front edges of the bands, but owing to the increased diffuseness the total separation may not be greatly increased in some cases. In any case it is clear that development should not be unduly prolonged.

An interesting feature of the above expression is that it is independent of the diameter of the column. This gives a definite advantage to a short wide column over a long narrow one owing to its greater practical convenience in working. This must not be overdone, however, as too rapid a solvent flow gives diffuse columns, due mainly to the fact that attainment of equilibrium is not instantaneous.

(4) Provided suitable conditions of quantity, concentration, partition ratio and degree of development are chosen, a single solute should give a band after development on a column with

(*a*) a sharp front edge, followed by
(*b*) a constant concentration zone, and
(*c*) the concentration in the solvent phase in equilibrium with the constant concentration zone should be equal to the concentration of the initial solution.

If a substance is found to give, for example, a band with a maximum near its top edge then it is not homogeneous. It is assumed, of course, that the banding on the column is regular.

Practical illustrations of the above considerations will be found in the subsequent papers of this series. They will apply *mutatis mutandis*, to all types of chromatogram except that, in the case of partition chromatograms of this type, the effect of one band on another will always be considerable because solution of the acids in the stationary phase will lower the pH. On the other hand, in many cases the direct adsorption of one substance by, for example, alumina is apparently not greatly affected by the adsorption of another.

Partition chromatograms of this type are clearly not limited to acids. With suitable buffers, bases should be separable (cf. Catch *et al.* 1942). It might also be possible, for example, to separate mixtures of aldehydes and ketones, using columns impregnated with bisulphite solution. The necessary conditions for a separation are clearly an adsorbed stationary phase and a moving phase, between which some or all of the substances to be separated can be differentially and reversibly distributed.

SUMMARY

1. A modified partition chromatogram for the separation of acids is described. It is based on that of Martin & Synge (1941), but concentrated potassium phosphate buffer solutions replace water as the stationary phase.

2. The theories of de Vault (1943) and Weiss (1943) are shown to give an adequate account of the behaviour of a single solute on such a chromatogram.

3. The effect of concentration on the distribution of phenylacetic acid or benzoic acid between diethyl ether and concentrated buffer solutions is expressible by an equation of the same form as the Freundlich adsorption isotherm.

Addenda (1947)

(1) The limits suggested for likely values of b in the equation $B=aC^b$ are too narrow. Acids have been encountered for which values of $b<0{\cdot}5$ have been found.

(2) The work originally intended to be published as part 3 in this series will be published in a forthcoming monograph on penicillin chemistry.

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Penicillin: Analysis of the Crude Product by Partition Chromatography

2. CHROMATOGRAPHIC ANALYSIS OF THE PENICILLINS FROM TWO STRAINS OF *PENICILLIUM NOTATUM*

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(*Received* 1 *September* 1947; *deposited with the Royal Society* 31 *October* 1944)

This paper is the second of a series dealing with the analysis of penicillin by means of a chromatogram using solvent-phosphate buffer partition on a silica base. The first paper (Levi, 1948) described the development of this type of chromatogram which is based on that of Martin & Synge (1941), and outlined the principles of its application to the study of penicillin.

The work described was carried out mainly in 1943, and communicated privately to collaborating laboratories in Great Britain and the United States at the end of July in that year. Wider publication was not possible at that time owing to wartime secrecy restrictions. It deals with an examination of the crude penicillin produced by two strains of *Penicillium notatum* grown under different conditions, and shows clearly that the main active constituents of the two products are different; a fact which was not appreciated until the work was carried out. The further examination of the crude product is also described and shows the existence of still other penicillins.

EXPERIMENTAL

Materials. Two types of crude penicillin were used. The first was prepared by growing a strain of *P. notatum* (Imperial Chemical Industries Ltd., ref. F4) obtained from Prof. H. W. Florey in surface culture on a Czapek-Dox medium with the addition of 0·5% acid-hydrolyzed casein. From the resulting broth the penicillin was isolated in the usual manner, by solvent extraction, as a calcium salt of activity 100–150 units/mg.

A sample of the second type was generously supplied to us by Merck and Co. Inc., Rahway, U.S.A. It was prepared by growing the pre-Fleming strain N.R.R.L. no. 832 (supplied by the Northern Regional Research Laboratory, U.S. Department of Agriculture) in submerged culture in a brown sugar-corn-steep liquor medium from which it was isolated as a sodium salt.

Biological assay. Two methods of assay were employed: a serial dilution method with *Staphylococcus aureus*, and a cylinder-plate method with *Bacillus subtilis* as the test organisms. In both cases a sample of calcium penicillin prepared from F4 strain penicillin was used as standard. In the case of the F4 material the activity determined by either method was the same, but with the 832 strain material the activity as measured by the *B. subtilis* method was significantly higher than when measured against *S. aureus*.

Treatment of crude penicillin with alumina. A preliminary treatment of the crude penicillin by this method greatly facilitated the subsequent chromatographic analysis by removal of much inert material. In a series of experiments it was found to raise the activity of the F4 strain material from 60–120 units/mg. to 260–330 units/mg., and in the case of the 832 strain material from 165 to 474 units/mg.

A typical experiment was as follows: crude calcium penicillin (22 g.) was dissolved in water (1·8 l.) and the solution was cooled in ice and stirred. Activated alumina (Type 'O', as supplied by Peter Spence and Sons Ltd., Widnes; 330 g.) was gradually added during 10 min., and stirring was continued for 1 hr. after which the alumina was filtered off and washed twice by stirring for 5 min. with 250 ml. water. The combined solution and washings were mixed with chloroform (2 l.), cooled to 3°, and the penicillin extracted into chloroform by acidifying to pH 2 with 4M-H_3PO_4 (60 ml.). The aqueous layer was quickly re-extracted with cold chloroform (1 l.). The extract, after washing with water, was stirred successively with three lots of water (300, 200, 100 ml.) containing precipitated chalk (30, 20 and 10 g. respectively). After filtration and separation the solution of calcium penicillin was dried from the frozen state. Weight of product, 6·47 g., 314 units/mg. (yield 81%).

Chromatographic analysis of penicillin preparations

Preliminary silica-phosphate buffer chromatogram of F4 strain penicillin. Silica (60–160 mesh; 185 g.) was mixed with 25% (w/w) potassium phosphate buffer (pH 6·27; 90 ml.) and diethyl ether (10 ml.) in a mortar. The powder was suspended in ethylene dichloride (1 l.) and stirred for 1 hr.; the resulting slurry was then poured into a long chromatogram tube containing a few cm. of ethylene dichloride. Solvent was allowed to run through the column until it ceased to shrink; the next day ether was run through until there was again no further shrinkage. The final dimensions of the column were 5·2 × 22 cm.

Alumina-treated calcium penicillin (8 g., activity 298 units/mg.) was dissolved in water (150 ml.), and, after acidification at 3° to pH 2, extracted into ether (250, 150, 100 ml.). The solution was run on the top of the column and, after it had sunk in, development was continued with ether until

most of the activity, as measured by the optical rotation in 2 dm. tubes, was found in the percolate which was divided into 5 fractions. The penicillin was isolated as barium salt from these by shaking the ethereal solution with a suspension of freshly precipitated $BaCO_3$, filtering, separating the aqueous layer, and drying from the frozen state. The residue on the column was recovered by suspending the silica in water (300 ml.); after stirring for 5 min. the suspension was filtered and the residue washed with water (200 ml.). The combined filtrate and washings were cooled below 5°, acidified to pH 2 with H_3PO_4 and the penicillin extracted with cold ether (250 and 150 ml.). The penicillin was then isolated as barium salt in the same manner as the main fractions. Results are given in Table 1.

Separation of the penicillins in the mixture from F 4 strain material. Early experiments suggested that the main active fraction obtained in chromatographic analysis (e.g. fractions 2–4 of Table 1) might be a doublet in which the slower moving constituent predominated. A consideration of the theory of the partition chromatogram (Lester Smith, 1942; Levi, 1948) suggested that if a larger band were the upper one it would tend to overtake a smaller one just below and thus render separation difficult. Accordingly column fractions at the head and tail of the main penicillin zone (such as fractions 3 and 5 in Table 1) were mixed and analyzed on a column 3·5 × 21·5 cm. made as above from silica (150 g.) and pH 6·5 buffer (70 ml.). The penicillin (3·0 g., activity 166 units/mg.) as calcium salt was transferred to ether (50 + 30 ml.) and applied to the column in the usual way; development was effected with ether (930 ml.). The results are given in Table 2. Fraction 2 (designated by us Penicillin V), which corresponded in properties with the first runnings from the column shown in Table 1, was shown to differ markedly from Penicillin I (fraction 5, Table 2) by a comparison of their partition coefficients between water and 25 % (w/w) potassium phosphate buffer. This comparison is shown in Table 3. The bands of the active material intermediate between fractions 2 and 5 we have designated penicillin IV.

Table 1. *Fractionation of F 4 strain penicillin by the partition chromatogram*

Fraction	Description of band	α_D (2 dm.)	Vol. of ether (ml.)	Wt. of salt (g.)	Activity (units/mg.)	Total units
1	Several pale yellow bands	−0·02°	540	0·42	12·5	5,200
2	Orange band (gives red colour with 5 % H_2SO_4 in acetic acid)	+0·05°	380	2·07	45	93,000
3	Very pale band, then yellow	+0·05° to +0·3°	250	0·45	198	89,000
4	Yellow, then mostly very pale	+0·5° to +0·25°	560	1·22	920	1,120,000
5	Very pale	+0·25° to +0·1°	600	0·35	733	266,000
6	Residue in a column	—	—	1·07	338	362,000
	Totals —	—	—	5·85 (70 %)	—	1,985,200 (81 %)

Table 2. *Penicillins I, IV and V*

Fraction	Vol. of eluate (ml.) or length of silica (cm.)	Weight (g.)	Activity (units/mg.)	Total units	Remarks
1	220 ml.	0·12	4	480	—
2	233 ml.	0·40	460	184,000	Pen. V
3	268 ml.	0·27	63	17,000	—
4	250 ml. + 4·0 cm.	0·25	419	105,000	Pen. IV
5	1·7 cm.	0·05	134	6,700	—
6	3·0 cm.	0·11	677	74,500	Pen. I
7	12·8 cm.	0·68	38·5	26,200	—
	Totals	1·88	—	413,880	—

Table 3. *Partition coefficients of penicillins I and V between ether and 25 % potassium phosphate buffer*

	pH ...	5·7	6·2	6·55	6·7
Pen. V	$\alpha \frac{\text{(conc. in buffer)}}{\text{(conc. in ether)}}$	0·35	1·8	3·1	8·6
Pen. I	$\alpha \frac{\text{(conc. in buffer)}}{\text{(conc. in ether)}}$	12	30	58	74

Table 4. *Preparation of penicillin III*

Fraction	Vol. of eluate (ml.)	Wt. of calcium salt (g.)	Activity (units/mg.)	Total units
1	100	0·08	17	1,360
2	50	0·16	18·5	2,960
3	50	0·18	39	7,030
4	50	0·18	40	7,200
5	50	0·12	83·5	10,000
6	50	0·12	335	4,020
7	Residue in column	0·08	4	320
	Totals	0·92	—	32,890

Penicillin remaining on the first column, such as fraction 6 (Table 1), was examined on a column of silica (50 g.) impregnated with 25 % (w/w) potassium phosphate solution of pH 5·0 (30 ml.) using 1·5 g. of material of activity 36 units/mg. Development was effected with ether (350 ml.). Detailed results are shown in Table 4.

Partition chromatogram of 832 *strain penicillin.* N.R.R.L. no. 832 strain calcium penicillin (0·6 g., activity 473 units/mg.) was transferred to ether in the usual way, and applied to a column (3·55 × 32 cm.) prepared from silica (115 g.) impregnated with pH 6·5 buffer (58 ml.). After development with ether the column was divided mechanically into fractions, and the penicillin was isolated in the usual way. The results are shown in Fig. 1.

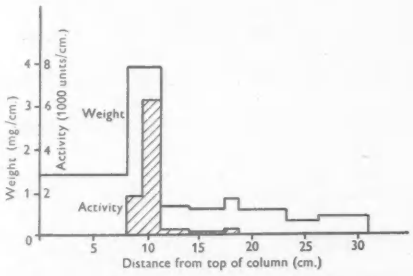

Fig. 1. Chromatogram of '832' penicillin.

Separation of mixed main fractions from F 4 *and* 832 *penicillins.* Sodium penicillin I fraction from F 4 penicillin, similar to fraction 5 of Table 2 (123 mg., activity 750 units/mg.) was mixed with the calcium salt of 832 penicillin (72 mg., activity 935 units/mg.) prepared as above and transferred to ether (40 ml.), and applied to a column (2·8 × 25·5 cm.) prepared from silica (73 g.) impregnated with pH 6·5 buffer (37 ml.). Development was effected with ether (850 ml.). The results are shown in detail in Fig. 2.

In order to provide a convenient scale for both column and eluate fractions the distances of movement of the bands are given in terms of their distance from the top of the column at the end of development. For those fractions which were eluted the distance is calculated on the assumption of uniform development in a very long column. If d cm.

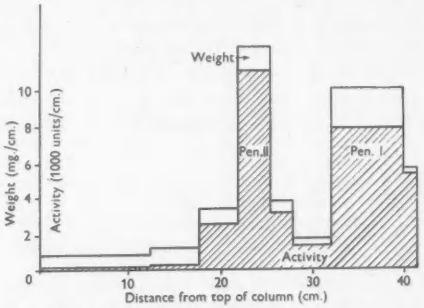

Fig. 2. Chromatogram of mixed 'F4' and '832' penicillins.

is the distance that such a fraction would have moved from the top of a column of actual length l cm., and v ml. is the volume of solvent required to develop it to the bottom of the column, and V ml. is the total volume of solvent used, then

$$d = \frac{Vl}{v}.$$

DISCUSSION

The behaviour of F 4 and 832 penicillins on a phosphate buffer chromatogram differed considerably. The former material gave a series of yellow or orange bands several of which fluoresced yellow or green in ultraviolet light. The main zone of antibacterial activity was rather diffuse, and was preceded by a deep, orange, practically inactive, band associated with the colour reaction of Catch, Cook & Heilbron (1942) and a small zone of weak antibacterial activity. There was also another small antibacterial zone above the main band. Further examination of the main zone revealed that it was composed of two substances of which one was present in considerably greater quantity than the other. This we have

designated as penicillin I. In order to demonstrate a definite separation from the minor constituent, designated penicillin IV, it was necessary to analyze chromatographically a mixture of the head and tail fractions of the main antibacterial zone since the large quantity of penicillin I relative to penicillin IV in the zone as a whole tended to swamp the latter.

The small zone of antibacterial activity, which came out with the first runnings from the first column, has been shown to be different from penicillin I both by separation from it on a partition chromatogram and by a direct comparison of the effective distribution coefficient of the two substances in bulk. It has been tentatively designated penicillin V.

The antibacterial substance, which remained on the first column, has been shown to be different from penicillin I by the fact that it was firmly retained on a column which would not retain penicillin I at all. The exact nature of this substance has not been determined owing to the small quantity available, but it is probably the same as the penicillin III which has been recognized in N.R.R.L. no. 1249 strain penicillin (Catch, Cook & Heilbron, 1944; Boon, Carrington & Levi, 1944).

The penicillin I isolated by the procedure described here had a potency of 800–1250 units/mg. and a purity of the order of 60%. The final purification of this material, by further chromatographic procedures followed by crystallization of the sodium salt, will be described in the forthcoming monograph on Penicillin Chemistry.

The significant difference between the biological activity of N.R.R.L. no. 832 strain penicillin against two different test organisms, when using an F4 strain standard, suggested that there was probably some difference in the constitution of the two substances. This was confirmed by chromatographic analysis. Not only was the general appearance, in visible and ultraviolet light, different from that of the F4 strain penicillin, but the main zone of activity was found to be much more compact and to develop more slowly than with the latter material. Final confirmation was obtained when a mixture of penicillin I and purified 832 penicillin was examined on a further column when a complete separation was observed. We have designated the new penicillin as penicillin II.

SUMMARY

1. Partition chromatography with a silica-phosphate buffer-solvent system has shown the existence of five penicillins.

2. The main antibacterial substance produced by the F4 strain of *Penicillium notatum* grown on a modified Czapek-Dox medium is penicillin I which is accompanied by small quantities of three other penicillins.

3. The main antibacterial substance produced by the N.R.R.L. no. 832 strain of *P. notatum* grown submerged in a brown sugar-corn-steep liquor medium is penicillin II different from any of the above.

4. There is a differential response of two organisms, *Staphylococcus aureus* and *Bacillus subtilis*, to the action of F4 penicillin and 832 penicillin.

Addendum (1947)

At the time this work was done in 1943 the chemical difference between penicillins I and II was unknown, and we introduced the system of nomenclature by which the penicillins, as discovered, were given a roman numeral. In modern notation penicillins I, II and III are respectively 2-pentenyl-, benzyl- and *p*-hydroxybenzyl-penicillin. The exact constitution of penicillin IV has not yet been determined, but the evidence available suggests that it is an isomer of 2-pentenylpenicillin. Penicillin V may be identical with *n*-heptylpenicillin.

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Studies on the Metabolism of Semen

4. AEROBIC AND ANAEROBIC UTILIZATION OF FRUCTOSE BY SPERMATOZOA AND SEMINAL VESICLES

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Previous studies on the metabolism of semen have furnished evidence that the reducing carbohydrate in seminal plasma is fructose, which is readily converted by the spermatozoa to lactic acid, thus providing an important source of energy for the sperm cells (Mann, 1946*a*, *b*, *c*). The rate of fructolysis represents an accurate and at the same time simple means of evaluation of semen; the fructolysis index (mg. fructose utilized by 10^9 spermatozoa in 1 hr. at 37°) in normal bull and ram semen is 1·4–2·0, while semen with poor sperm motility gives much lower index values; azoospermic and necrospermic semen was shown to be altogether unable to metabolize fructose (Mann, 1948*a*, *b*). Fructose originates in the accessory glands of reproduction, mainly the seminal vesicles, but in some species it is found also in the ampullae and in certain parts of the prostate organ (Mann, 1946*c*; Davies & Mann, 1947). The process of fructose formation is initiated and controlled by the testicular hormone; a hormonal deficiency due, for example, to castration, causes invariably a decrease or disappearance of seminal fructose, but treatment with testosterone promptly restores the ability of the accessory glands to produce fructose (Mann & Parsons, 1947).

In this paper it will be demonstrated that fructolysis is a characteristic feature of both the aerobic and anaerobic metabolism of semen. Unlike spermatozoa, the seminal vesicles will be shown to lack the ability to utilize fructose anaerobically. Finally, the position will be discussed of fructolysis in relation to glucolysis and respiration, in both semen and seminal vesicles.

EXPERIMENTAL

Material. Ram semen was obtained by the method of Walton (1945); the procedure for washing the spermatozoa and the composition of the special Ringer solution were the same as previously described (Mann, 1945*a*, *b*, *c*). Seminal vesicles from fully fertile rats were slit open along the outer edge and washed in Ringer solution to remove the secretory fluid. Portions of the basal parts of the organ (8–10 mg., dry weight) were used so as to include as much as possible of the 'coagulating gland', which in rats contributes, together with the dorsal prostate, the bulk of seminal fructose. Seminal glands from bulls were collected from freshly slaughtered animals.

Methods. Three methods were used for the assay of fructolysis: (1) manometric estimation of acid production was carried out in Barcroft differential manometers with gas outlets, by measuring the CO_2 output using Ringer-bicarbonate and a gas mixture of 95 % N_2 and 5 % CO_2; (2) lactic acid was estimated by the method of Friedemann, Cotonio & Shaffer (1929); (3) fructose was determined colorimetrically as described before (Mann, 1948 *a*, *b*). Respiration was measured manometrically in air and Ringer-phosphate.

RESULTS

Utilization of fructose by sperm

Anaerobic fructolysis. Certain points which emerged from the study of fructolysis in washed spermatozoa are illustrated in Fig. 1. The experiment recorded in this figure was carried out with suspensions of washed spermatozoa of ram in Ringer-bicarbonate solution, incubated anaerobically by shaking at 37° in Barcroft manometers filled with 95 % N_2 and 5 % CO_2. In each case the final volume of the mixture in the manometric flask was 2·5 ml. The following mixtures were used: I, $0{\cdot}65 \times 10^9$ cells + 1·9 mg. fructose; II, $0{\cdot}65 \times 10^9$ cells + 1·9 mg. glucose; III, $0{\cdot}13 \times 10^9$ cells + 1·9 mg. fructose. In I and II the acid formation followed the course of a straight line so long as there was still a little sugar left; after 110 min. incubation the final yield of CO_2 was in I: 420 μl. CO_2, corresponding to 1·68 mg. lactic acid; in II: 410 μl. CO_2, corresponding to 1·64 mg. lactic acid. By chemical analysis of lactic acid, 1·63 mg. was obtained in I, and 1·58 mg. in II. Estimation of sugar showed that only 0·05 mg. fructose was left in I, and 0·07 mg. glucose in II. Taking the 60 min. value as the basis for the calculation of the metabolic rate, one finds that 290 μl. CO_2 or 1·16 mg. lactic acid has been produced from fructose or glucose alike by $0{\cdot}65 \times 10^9$ sperm cells contained in mixtures I and II, i.e. 1·78 mg./10^9 sperm/hr. However, in III the acid amounted to not more than 24 μl. $CO_2/0{\cdot}13 \times 10^9$ sperm/hr., that is only 0·77 mg./10^9 sperm/hr.

Aerobic fructolysis and O_2 *consumption.* Aerobic experiments were carried out with the same ram semen as above, but the suspensions of washed spermatozoa contained phosphate instead of bicarbonate; the amounts of sugar added were the same as before. The O_2 uptake in suspensions I and II can be seen to follow a linear course throughout the period of 110 min. (Fig. 2). However, whereas in the course of anaerobic activity (Fig. 1), suspensions

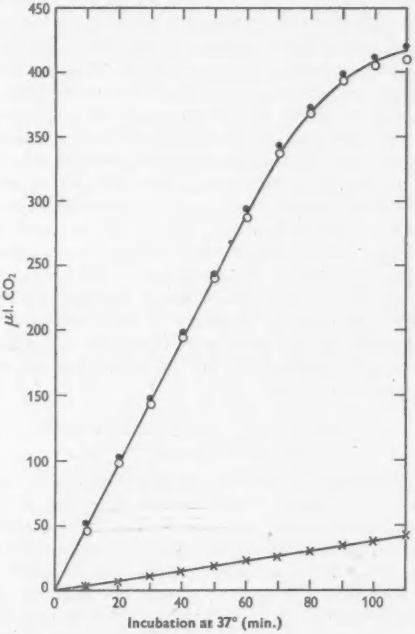

Fig. 1. Anaerobic fructolysis and glucolysis by washed spermatozoa. I: ●——● $0{\cdot}65 \times 10^9$ ram sperm + fructose; II: ○——○ $0{\cdot}65 \times 10^9$ ram sperm + glucose; III: ×——× $0{\cdot}13 \times 10^9$ ram sperm + fructose.

I and II used up nearly all the sugar, in the corresponding aerobic experiment (Fig. 2), out of 1·9 mg. sugar added, 0·42 mg. fructose and 0·48 mg. glucose, respectively, were found to be still intact after 110 min. In the same samples lactic acid was determined; 1·03 and 0·98 mg. lactic acid were found at the end of 110 min. respiration period in I and II respectively. It can be seen from Fig. 2 that the sperm suspensions I and II, each containing $0{\cdot}65 \times 10^9$ sperm/2·5 ml. Ringer solution, consumed 130 μl. O_2 in 1 hr. or 200 μl. $O_2/10^9$ sperm/hr. However, suspension III which is five times more diluted than I and II ($0{\cdot}13 \times 10^9$ sperm/2·5 ml. Ringer solution) consumed only 15 μl. O_2 or 115 μl. $O_2/10^9$ sperm/hr. The low rate of respiration in III recalls the previously mentioned low rate of anaerobic

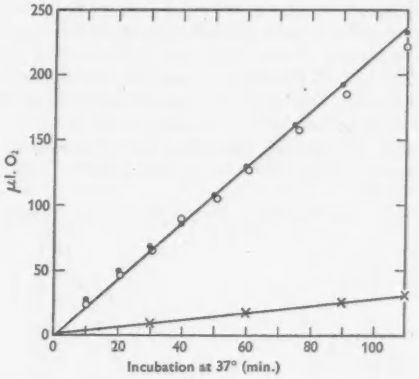

Fig. 2. Oxygen consumption of ram spermatozoa. I: ●——● $0{\cdot}65 \times 10^9$ ram sperm + fructose; II: ○——○ $0{\cdot}65 \times 10^9$ ram sperm + glucose; III: ×——× $0{\cdot}13 \times 10^9$ ram sperm + fructose.

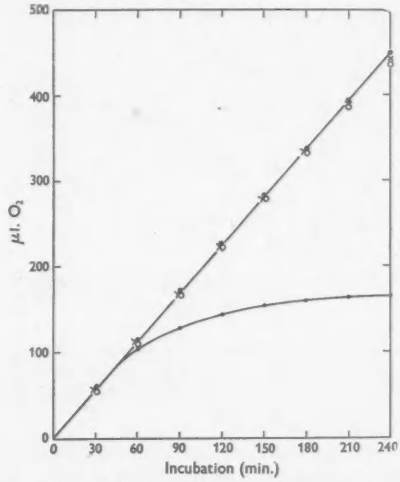

Fig. 3. Effect of fructose, glucose and lactate on the respiration of washed spermatozoa. 1 ml. ram semen diluted with 3 ml. Ringer solution, centrifuged, sperm washed with 5 ml. Ringer solution, resuspended in Ringer solution and diluted to 16 ml. Each manometer flask contained 2 ml. sperm suspension, corresponding to $0{\cdot}45 \times 10^9$ sperm cells, and 0·5 ml. isotonic phosphate buffer with: ·——· no additions; ●——● 1 mg. fructose; ○——○ 1 mg. glucose; ×——× 1 mg. lactic acid (as Na lactate).

glycolysis and must be attributed to the same cause: the injurious effect of dilution on spermatozoa.

It has been known that ram spermatozoa freed from seminal plasma, and thus deprived of glycolyzable material, retain their ability to consume oxygen at an almost normal rate of respiration of about 200 μl. $O_2/10^9$ sperm/hr. (Lardy, Winchester & Phillips, 1945; Mann, 1945 *c*). Thus the aerobic metabolism in spermatozoa appeared independent of fructolysis. However, in the present study, with suitable dilutions of washed spermatozoa, it was found that the O_2 uptake in such suspensions remained constant only for a limited period of time,

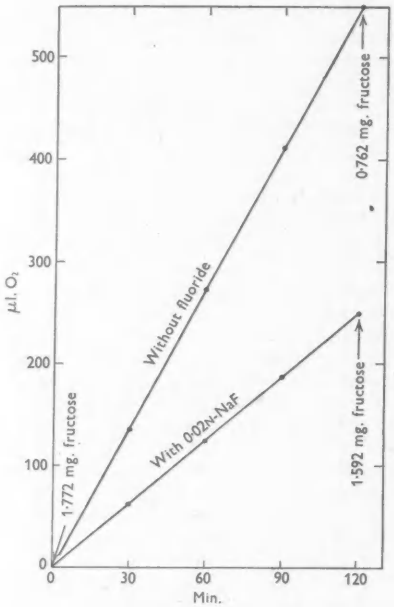

Fig. 4. Effect of fluoride on the respiration and aerobic fructolysis of ram semen. Each manometer flask contained 0·5 ml. whole semen diluted with 1·5 ml. Ringer-phosphate solution.

and that during the later stage it declined progressively unless the sperm were provided with an additional source of oxidizable material such as fructose, glucose or lactate. Their effect on the O_2 uptake of washed spermatozoa is illustrated by Fig. 3, whence it can be seen that all three of them maintained equally well the initial rate of respiration for a considerable length of time, although they did not raise significantly the initial rate of O_2 consumption. On the other hand, if added to a respiring suspension of washed spermatozoa at a later stage, when the respiration had already begun to decline, they prevented further deterioration in the rate of O_2 consumption.

In order to explore the significance of glycolysis in the aerobic metabolism and survival of spermatozoa, use was made of certain substances such as iodoacetate and fluoride which were previously shown (Lardy & Phillips, 1943) to interfere with sperm activity. Iodoacetate proved to be a rather strong inhibitor not only of fructolysis but of the sperm respiration as well. More interesting results were achieved with fluoride. It was possible, using a suitable concentration of NaF, to suppress the fructolytic activity of whole semen much more strongly than the respiration. This can be seen from Fig. 4, which shows the O_2 uptake of two identical samples of semen, one treated with fluoride, while the other served as a control. In both these samples fructose was estimated at the beginning of the experiment and then after 2 hr. aerobic incubation in Barcroft manometers at 37°. The results of fructose analyses are indicated by arrows on Fig. 4. It can be calculated from these values that 0·5 ml. untreated whole semen consumed in 2 hr. 550 μl. O_2 and 1·01 mg. fructose, whereas the corresponding fluoride-treated sample utilized in the same period 250 μl. O_2, but only 0·18 mg. fructose. When at the end of a 2 hr. period the motility of the sperm was examined, the untreated spermatozoa were found to be perfectly motile, but those in the fluoride-treated semen were mostly immotile.

Fructose and glucose utilization in the seminal vesicle

It was shown in the foregoing that spermatozoa are cells capable of utilizing fructose and glucose both anaerobically and aerobically. In this respect they differ from most other animal tissues; the latter are able to metabolize fructose aerobically, but seldom show themselves capable of metabolizing it anaerobically at a rate comparable to that of glucose (Dickens & Greville, 1932, 1933). A study of carbohydrate metabolism in the seminal vesicle of the rat gave the following results, illustrated in Fig. 5. The anaerobic acid production in the seminal vesicles was found to be equally low in the absence as in the presence of added fructose. However, when glucose was added instead of fructose, it increased very strongly the rate of anaerobic glycolysis in seminal vesicles. It should be pointed out, however, that even the increased rate of glycolysis, as observed in the seminal vesicle in presence of glucose, was below the rate of glycolysis of spermatozoa. Assuming that the average rate of glycolysis in semen is 1·7 mg. sugar or 425 μl. $CO_2/10^9$ sperm/hr. and that the dry weight of 10^9 sperm is 30 mg., we arrive at the value of $Q_L^{N_2} = +14{\cdot}2$ for the glycolysis quotient of spermatozoa. The seminal vesicle tissue, on the other hand, has been shown to produce anaerobically 2·3 μl. CO_2/mg. dry wt./hr. and thus to have a quotient $Q_L^{N_2} = +2{\cdot}3$.

In view of the marked differences in anaerobic metabolism which exist between the sperm cells and seminal vesicles the possibility was considered that similar differences may also prevail under aerobic conditions. Seminal-vesicle slices incubated aerobically respired much better in presence of additional sugar, and the increase brought about by fructose was, if anything, even higher than that due to glucose (Fig. 6). The Q_{O_2} values for rat seminal-vesicle slices, calculated from the data of Fig. 6, are $-5{\cdot}3$ in presence of fructose, $-4{\cdot}2$ in presence of glucose, and $-2{\cdot}0$ in absence of added sugar. Even

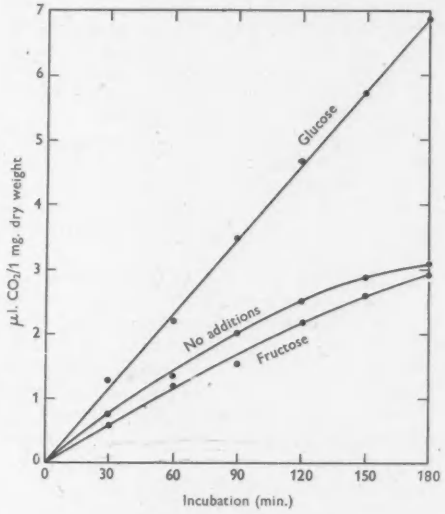

Fig. 5. Anaerobic fructolysis and glucolysis in rat seminal vesicle.

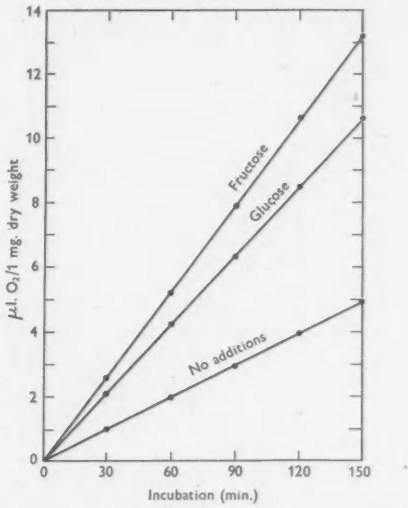

Fig. 6. Respiration of rat seminal vesicle in presence of fructose and glucose.

the highest of the three values is far below the Q_{O_2} of washed spermatozoa. Taking Fig. 3 as the basis of calculations, the O_2 uptake of ram sperm amounts to 112 μl. $O_2/0{\cdot}45 \times 10^9$ sperm/hr., i.e. 250 μl. $O_2/10^9$ sperm/hr. The dry weight of 10^9 sperm cells represents some 30 mg. so that the Q_{O_2} of spermatozoa is $-8{\cdot}3$.

Bull seminal glands were investigated in the following manner: the fresh gland was divided into two symmetrical portions, in one of which fructose was estimated at once, while the other was incubated at 37° for 2 hr. The level of fructose in the incubated sample did not differ markedly from the control. Similar results were obtained when the glands were minced or extracted with water and the pulp or aqueous extract used for incubation. A few experiments were also carried out in which minced seminal glands, slices or extracts, were incubated in presence of added carbohydrates. In presence of glucose, aerobically, a formation of free fructose was observed in slices, but the quantities were small. On the other hand, considerable quantities of 6-phosphofructose, together with 6-phosphoglucose, were formed from glycogen and Cori ester, by dialyzed and non-dialyzed extracts; but there was no increase in free fructose or lactic acid. It was also found that when dialyzed extract prepared from seminal vesicles was added to an actively glycolyzing Meyerhof muscle extract, it checked the formation of lactic acid from glycogen and led to the accumulation of 6-phosphohexose esters. It should be pointed out, however, that the extracts from seminal glands contain very active phosphatases which dephosphorylate adenylic acid, adenosine triphosphate and cozymase. Thus it is probable that the effects described above are at any rate partly due to the inactivation of the glycolytic coenzymes.

DISCUSSION

In bull and ram semen the anaerobic survival of spermatozoa is closely dependent upon the presence of fructose. If ram or bull spermatozoa are washed free from seminal plasma, and thus deprived of fructose, they soon become immotile under anaerobic conditions. But their survival can be extended considerably by the addition of glycolyzable sugar. So far, there is no evidence which would point to the existence of other anaerobic processes in semen,

capable of supplying the spermatozoa with metabolic energy on a scale comparable with fructolysis. Under aerobic conditions, on the other hand, spermatozoa can survive temporarily, even after the removal of seminal plasma. However, our experiments on washed ram spermatozoa showed that their ability to take up oxygen is of relatively short duration, but that it can be maintained for a considerable length of time by the addition of certain substances. Among these are fructose and lactate, both of which are normally present in semen. These findings lead to the conclusion that the metabolism of fructose plays an important role not only in the absence of oxygen but also in aerobic conditions. It remains for further study to ascertain precisely what type of metabolism, aerobic or anaerobic, predominates in spermatozoa during their existence in either the male or female genital tract. However, so far as *in vitro* storage is concerned, such as, for instance, for the purpose of artificial insemination, there is little doubt that the predominant process which supplies the vital energy to the cells is fructolysis and not respiration (Mann, 1948 *a*, *b*).

Although fructose is the 'physiological' carbohydrate, and there is practically no glucose in whole semen, yet spermatozoa which have been washed free from seminal fructose are capable of utilizing added glucose to the same extent as fructose. This behaviour of spermatozoa is in striking contrast to that of most other animal tissues, including the seminal vesicles which in most species produce the bulk of seminal fructose. This tissue utilizes glucose anaerobically, but not fructose. Aerobically, however, the seminal vesicles can utilize both glucose and fructose, and in this respect they do not differ from spermatozoa.

SUMMARY

1. The process of fructolysis plays an essential role in the survival of mammalian spermatozoa both under anaerobic and aerobic conditions. Anaerobic fructolysis provides the main source of energy for spermatozoa. Aerobically both fructose and lactic acid function by prolonging and maintaining the sperm respiration.

2. In buffered sperm suspensions fructolysis continues at a steady rate, and follows a linear course until practically the entire sugar content has been exhausted. The rate of fructolysis is greater under anaerobic than aerobic conditions. Excessive dilution of sperm suspensions causes a decrease in the rate of both fructolysis and respiration.

3. By using a suitably chosen concentration of sodium fluoride it is possible to abolish fructolysis completely while the respiration is only partly suppressed. Under such conditions it was found that the spermatozoa were immotile even although there was still some O_2 consumption.

4. Spermatozoa washed free from fructose-containing seminal plasma can utilize anaerobically to the same extent added fructose or glucose. In this respect the sperm cells differ from the fructose-producing tissue, the seminal vesicle, which utilizes glucose anaerobically, but not fructose. Aerobically, however, both sperm and the seminal vesicles are capable of metabolizing both fructose and glucose.

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Tropomyosin: a New Asymmetric Protein Component of the Muscle Fibril

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Although the characterization of the proteins in muscle is by no means complete, it is generally supposed that the major component of the myofibril is the myosin filament, and that the proteins of the sarcoplasm are largely enzymic in function. There is no reason seriously to doubt this view, but, in point of detail, the composition of the fibril is of considerable complexity. Liubimova & Engelhardt (1939) first showed that isolated myosin is associated with adenosine triphosphatase activity, and subsequent attempts to separate the enzyme from myosin have been unsuccessful. The actin component of Straub (1942, 1943), studied in some detail by Szent-Györgyi and the Szeged school, must also be considered as part of the fibril. Indirect assay suggests that the proportions of myosin and actin are in the ratio 8 : 3 (Balenović & Straub, 1942). Actin possesses the property of converting myosin sols into a thixotropic gel, an interaction which involves the sulphydryl groups of the myosin partner (Bailey & Perry, 1947*a*, *b*).

In the present paper, a new fibrillar protein of asymmetric character and relatively low molecular weight, quite distinct in all its major properties from actin, is described. A brief report of its isolation has already been published (Bailey, 1946*a*). Whilst the emphasis in considering the function both of adenosine triphosphatase and of actin has been upon their possible role in the contractile process, the properties of this newer component suggest that it is a prototype of the much larger myosin molecule and might conceivably be a unit utilized in the elaboration of myosin itself. The evidence for this view derives partly from a consideration of its physical properties and amino-acid composition, but more particularly from its relation to the keratin-epidermis-myosin-fibrinogen (*k-e-m-f*) group as revealed by X-ray and electron optical studies, reported by Astbury, Reed & Spark (1948) in an accompanying paper. The protein has been designated *tropomyosin*, not so much to describe its function, which as yet is tentatively proposed, but to imply that in all its properties it is a prototype of myosin. Although intrinsically asymmetric, it crystallizes from dilute salt solutions to give fragile birefringent plates containing some 90% of their weight as water; in absence of salt, its viscosity increases enormously, due to a spontaneous aggregation of molecules into fibres of regular width and somewhat variable length.

EXPERIMENTAL

Preparation of tropomyosin

Stage 1. Freshly excised rabbit muscle is minced, mixed with an equal volume of water, and homogenized for 1 min. in a Waring Blendor. After standing for 30 min., the juice is squeezed out in a hand press and an equal volume of ethanol added to the residue. The liquor is again expressed and the debris treated with 4 vol. of 1 : 1 ethanol-water. The ethanol washing is continued through two changes of 97% ethanol and then through two of ether. The fibre is dried in air at room temperature and, while still ether-damp, is immersed in M-KCl (700 ml./100 g. fibre), and the pH adjusted to 7 with N-NaOH. After 12 hr. at 15–20°, the supernatant liquid appears viscous and is expressed through muslin. The residue is now re-extracted as before with M-KCl for 1–2 hr. and the liquid squeezed out in a hand press.

Stage 2. The combined unfiltered extracts are acidified to approx. pH 4·3 with N-HCl (yellow-green to bromocresol green), and after 1 hr. the precipitate is spun down, leaving a clear supernatant fluid. The precipitate is dispersed in 5 vol. of water and the pH readjusted to 7 with N-NaOH. At this stage, part of the protein originally soluble remains as a suspension of denatured material, whilst the tropomyosin passes into solution. Denatured protein, together with fibre debris, is removed in the next step.

Stage 3. The total volume is measured and 0·7 vol. of saturated $(NH_4)_2SO_4$ (containing 0·01 vol. of conc. ammonia solution) is added with rapid stirring. The flocculum of denatured protein is centrifuged down (or filtered), leaving a clear supernatant liquid (or filtrate) to which solid $(NH_4)_2SO_4$ is added in portions to 70% saturation. The precipitate of tropomyosin is filtered off under gravity, allowed to drain overnight, dissolved in water and dialyzed for approx. 24 hr. against a large volume of water.

Stage 4. The dialyzed sol is treated with N-HCl to the point of maximal precipitation (near pH 4·5), the precipitate spun down, dispersed in water and neutralized to pH 7. The salting-out procedure of stage 3 is now repeated, with the difference that the initial $(NH_4)_2SO_4$ concentration is taken beyond 41% saturation until a slight turbidity develops (45–47% saturation). This precipitate of tropomyosin plus impurities is discarded and the supernatant liquid brought to 70% saturation. The tropomyosin is filtered off, dissolved, dialyzed, and once more subjected to the purification procedure of stage 4.

Stage 5. The final precipitate of tropomyosin obtained by salting out to 70% saturation is dissolved in water, filtered through paper-pulp, dialyzed at 0° for 2 days against many changes of distilled water (maintaining the pH inside the sac at 6·5), and finally against 0·1 M-KCl. (The absence of free ammonium ions is checked by precipitating a portion of the sol with trichloroacetic acid and determining the N content of the filtrate.) The presence of KCl assists the subsequent precipitation with ethanol (effected with 1·5 vol.). The precipitate is dried through several changes of ethanol and then with ether. The dehydration of the protein is slow and is facilitated by pressing the gel particles on a sintered glass filter.

When the protein is required for re-solution, it may be stored (preferably at 0°) after drying as above; alternatively, it may be kept at 0° in the form of an $(NH_4)_2SO_4$-containing paste after a salting-out procedure. When required for analysis, it is preferable to precipitate from a dialyzed sol by addition of dilute acetic acid to pH 5; such precipitates, after drying as before, contain only traces of ash.

Purity of the preparation. The homogeneity of tropomyosin thus prepared was checked by electrophoresis (see below) and by ultracentrifugal analysis (Bailey, Gutfreund & Ogston, 1948). More rapid tests of purity, however, are available: (1) the glyoxylic reaction for tryptophan, using an equal weight of myosin as control, is extremely faint or negative; (2) a 2% sol in water (pH 6·5) is water-clear: any dispersed, denatured protein imparts an opalescence and tends to precipitate on heating in 0·1 M-potassium chloride; (3) in absence or presence of salt (e.g. 0·5 M-potassium chloride), the protein dissolves in hydrochloric acid below pH 4 to give a clear solution.

Crystallization of tropomyosin. The protein readily crystallizes, even from impure solution. A 2–3% solution (pH 6·5–7) is dialyzed at 0° against a salt solution containing 16 g. ammonium sulphate/l. and 0·01 M with respect to acetate buffer pH 5·4. When the pH of the sol reaches 5·8–6·0, crystals are deposited in large clusters on the cellophan membrane and drop into the bottom of the sac. The various forms observable are depicted in Pls. 4 and 5 for skeletal rabbit muscle and pig cardiac muscle, but similar crystals have been obtained from teleost (whiting) muscle and from horse heart. In polarized light they are strongly birefringent, but in ordinary light they are difficult to view or to photograph because of their transparency. They are extremely fragile, and the larger crystals tend to break if stirred or touched.

In practice, it is often convenient to employ for crystallization the salted-out precipitate described in the method of preparation. This is filtered off under gravity, allowed to drain overnight, mixed to a slurry with an equal volume of water, dialyzed (at constant volume) first against water until complete solution, then against several changes of the outer liquid described above. By retarding the rate of change of pH inside the sac (either by using small volumes of outer liquid or infrequent changes of larger volumes) crystallization can be regulated to give large crystals.

Crystallization appears to depend upon the presence of sufficient salt to break up the large aggregates which are formed spontaneously in salt-free solutions (see below); these unit molecules are then amenable to isoelectric crystallization. Crystals cannot be obtained either from pure water or from concentrated salt solutions. As a purification process, crystallization is quite inadequate, since tryptophan-containing protein may still be detected in preparations three times crystallized. The composition of crystals obtained from material first purified by the methods already outlined, followed by three recrystallizations, appears identical with the original homogeneous, uncrystallized specimen (Table 1). Electrophoresis likewise gave proof of their homogeneity.

Table 1. *Composition of a three-times crystallized rabbit tropomyosin compared with uncrystallized homogeneous tropomyosin*

(Results* as percentage of protein weight.)

	Total N	Amide N	Tyrosine	Tryptophan
Homogeneous uncrystallized	16·7	0·89	3·2	*c.* 0·02
Homogeneous crystallized	16·7	0·90	3·1	*c.* 0·02
Unpurified crystallized	16·9	0·85	2·9	*c.* 0·05

* See analytical section for methods of analysis.

A quite unique property of the crystals is their enormous hydration. After sucking off excess mother liquor and pressing between filter paper, two different batches of crystals gave the following analysis:

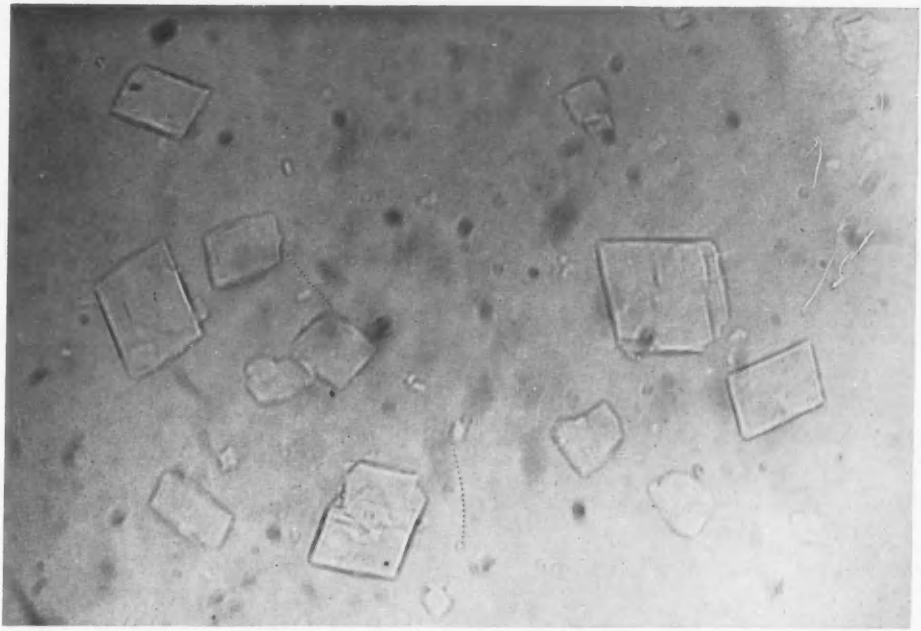
Sample 1: water, 86·9; ammonium sulphate, 2·0; protein, 11·1%.

Sample 2: water, 89·6; ammonium sulphate, 1·74; protein, 8·7%.

By comparison, lactoglobulin (McMeekin & Warner, 1942) contains 45·6 g. and edestin (Bailey, 1942 *a*) *c.* 40 g. of water/100 g. of wet crystal.

Assay of tropomyosin

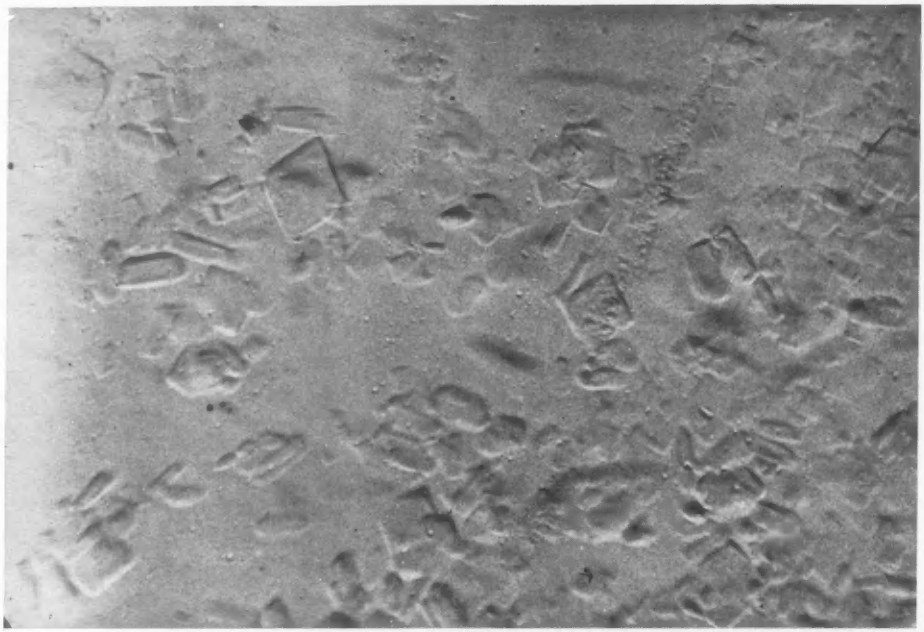
The method comprises essentially stages 1, 2 and 3 described in the preparation. The incorporation of certain modifications necessitates a résumé of the steps involved: (*a*) The minced muscle (100 g.) is homogenized for 1 min. with an equal volume of water in a Waring Blendor, and squeezed out in muslin after addition of 200 ml. ethanol. The residue is dried in ethanol and ether as before. (*b*) It is then extracted with 100 ml. M-KCl at pH 7, first for 12 hr. at 15–20°, then for 3 hr., again for 3 hr., and washed finally with a further change of KCl solution. (*c*) The protein in the combined extracts is precipitated at pH 4·3 with N-HCl, centri-



(×350)

(×490)

Tropomyosin crystals from rabbit skeletal muscle.



(*a*) (×280)

(*b*) (×560)

Tropomyosin crystals from pig heart muscle: (*a*) ordinary light; (*b*) polarized light.

fuged, the precipitate dispersed in water (80 ml.), neutralized to pH 7 and treated with saturated $(NH_4)_2SO_4$ solution (containing 0·01 vol. conc. ammonia solution) to 41% saturation. The precipitate, after centrifuging, is triturated with 2 parts of 41% saturated $(NH_4)_2SO_4$ solution, and again spun down. The combined supernatant fluids are treated with solid $(NH_4)_2SO_4$ to 85% saturation, and the precipitated protein filtered off under gravity. It is then dissolved in 100 ml. water and dialyzed for 4 days against frequent changes of 0·1 M-KCl solution. (*d*) To a 10 ml. sample of the dialyzed sol is added 0·2 ml. M-phosphate, pH 6·3, and the solution heated in a boiling water bath for 10 min. After cooling for 1 hr. the slight precipitate of denatured protein is spun down, leaving tropomyosin in solution. The protein content is determined by estimating the total N and correcting for free NH_3 by distilling an undigested portion of sol at pH 9·5. The protein N content × 6 gives the yield of tropomyosin.

Prepared in this way, the protein appears to be quite pure; the heat-denatured impurity of step (*d*), for example, gives a pronounced tryptophan reaction, whilst the tropomyosin isolated from the supernatant fluid only feebly responds.

The hind-leg plus back muscle of adult rabbits gave yields of 0·42, 0·50, 0·50, 0·48, 0·46 g./100 g. fresh muscle weight, each value being obtained from a different animal. The mean value (0·47 g./100 g.) comprises some 2·6% of the total protein and 4–5% of the myosin fraction. One determination on the cardiac muscle of the pig gave a lower value of 0·21 g./100 g. muscle.

Tropomyosin as a naturally occurring constituent of muscle

The conditions for the preparation and assay of tropomyosin have evolved from a series of preliminary experiments directed towards the elucidation of three important points: (1) that the protein does not arise by post-mortem catheptic breakdown; (2) that it is not produced by the disaggregating action of salt solutions upon ethanol-denatured myosin; (3) that it is a constituent of the fibril and not of the sarcoplasm. These points will be considered in turn.

Tropomyosin and post-mortem changes. Muscle tissue is characteristically poor in proteolytic enzymes (Fearon & Foster, 1922; Smorodinzew, Schirokow & Zyganowa, 1933), but even so, the exclusion of post-mortem changes was thought necessary. Homogenized muscle, obtained some 7 min. after death, was divided into three portions; the first (*A*) was treated immediately with 1 vol. ethanol, squeezed and dried in the usual way; the second (*B*) was allowed to stand at 20° for 4 hr. before addition of ethanol; the third (*C*) was washed immediately in three changes of water, and after standing for 4 hr. was treated with ethanol. Tropomyosin was then estimated in all three residues.

The yields in all cases were quite comparable (Table 2). The arrest of proteolysis as in (*A*), or the removal of soluble enzymes as in (*C*), does not reduce the yield below that of (*B*) in which rigor changes are allowed to proceed.

Table 2. *Yield of tropomyosin from homogenized rabbit muscle*

Animal	Treatment of homogenate*	Yield of tropomyosin (g./100 g. fresh muscle)
Rabbit *DX*	*A*	0·52
	B	0·45
	C	0·51
Rabbit *FX*	*A*	0·52
	B	0·48

* *A*, treated with ethanol 7 min. after death; *B*, treated with ethanol 4 hr. after death; *C*, washed thrice with water and treated with ethanol 4 hr. after death.

The possible disaggregating effect of salt solutions in the production of tropomyosin. This second possibility was explored in two ways, by studying the action of salts on (*a*) ethanol-dried muscle, and (*b*) on ethanol-dried myosin. In the first case, it was shown that three successive extractions with M-KCl suffice to exhaust the residue at a point where the total protein extracted is only some 4·5% of the myosin remaining in the residue; a fourth overnight extraction increased the yield by only 2%.

Likewise, there is no evidence of disaggregation when myosin itself is treated in a similar manner. In this case, thrice precipitated myosin, prepared by the method of Bailey (1942*b*), was freeze-dried and extracted with ethanol and ether. The residue (8·8 g.) after three extractions with M-KCl gave, in the fourth extract, a mere trace of material precipitable by trichloroacetic acid. The combined extracts yielded on appropriate treatment a small amount of material which, if tentatively considered as tropomyosin, amounted to 0·1% of the myosin taken. (It may be noted here that M-KCl reduces the P content of dried myosin, due to the extraction of nucleic acid P.)

Tropomyosin as a component of the fibril. It has already been demonstrated (Table 2) that water-extracted muscle mince is not depleted of tropomyosin, and that in consequence the histological site of the protein must reside either in the fibril, the collagenous supporting structures, or in the nuclear material. The evidence favours the idea that tropomyosin is of fibrillar origin. As will be shown later, tropomyosin must be considered, both chemically and structurally, a species of myosin, and is thus most likely to occur with myosin. Moreover, though a water-soluble protein, it is extracted from muscle mince only under conditions which effect the dispersal of myosin (i.e. in 0·5–1·0 M-KCl), and then only slowly and incompletely. This dispersal is greatly facilitated by denaturing the fibrillar proteins in ethanol, and even then, salt solutions are necessary for extraction. In these phenomena we have a clear indication that *in situ* tropomyosin is fixed to the structural proteins, possibly by electrostatic forces arising from the large numbers of base and acid groups which it contains. The efficacy of the ethanol-ether treatment might suggest in addition an association with lipids. Whatever the mode of combination, its liberation seems to depend upon a process of metathesis in presence of salt, a reaction simulative of that between histone and thymonucleic acid. For this reason, the conception of tropomyosin as a constituent of the nucleus cannot altogether be dismissed, but is improbable since the amount of thymonucleic acid, which may be

accepted as a guide to the total amount of nuclear matter, is only *c.* 0·04–0·06% (Davidson & Waymouth, 1944; Schneider & Klug, 1946).

The data relating to some of these observations may now be given. In Fig. 1 is shown the efficacy of water and salt solutions in extracting tropomyosin from ethanol- and ether-dried muscle mince. The

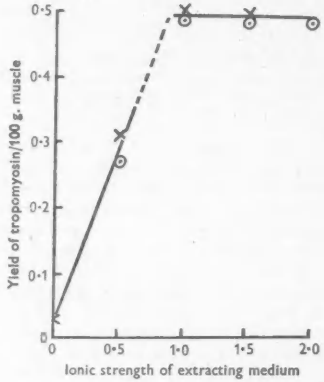


Fig. 1. The yield of tropomyosin from minced, ethanol- and ether-dried rabbit muscle as a function of the ionic strength of the extracting medium (KCl). The two sets of points relate to two different rabbits.

amount extracted by water is nil, and that by salt solutions a linear function of molarity up to 1·0 M, when the yield is maximal. If, on the other hand, freshly minced muscle is similarly extracted with salt solutions, the conditions here being optimal for extraction of myosin, the residues, after drying in ethanol and ether, still yield considerable amounts of tropomyosin; extraction with 0·5 M-potassium chloride leaves 80% of the total, and with 1·0 M 20%, in the residue. The molarity of the salt solutions is again seen to be important in the case of fresh muscle, but even in M-potassium chloride, extraction is incomplete. The use of ethanol-dried mince not only facilitates extraction, but gives an extract free from myosin and other soluble proteins. It may be noted that the acetone-dried muscle residue used in the preparation of actin (Straub, 1942) still contains tropomyosin after the actin has been extracted.

Properties of tropomyosin

Solubility. Above pH 6·5 and below pH 4·5, tropomyosin is soluble in water and in dilute salt solutions; the zone of complete insolubility in 0·01 M-sodium chloride extends from pH 4·5 to 5·3. The rate of settling of the precipitated particles is most rapid at pH 5·1, indicating that the isoelectric point lies near this pH value. In neutral ammonium sulphate solutions there exists a wide precipitation zone between the limits 45–60% saturation.

Denaturation. In 0·1 M-salt solutions, the protein is not precipitated by heating at 100° at pH > 6·3; the bulky precipitate which exists in the isoelectric zone transforms on heating to a discrete flocculum which again disperses at pH 7, not to a true solution, but to a translucent gel. After heating, or after dissolving in strong urea solutions, the protein no longer crystallizes. On the other hand, dehydrating solvents do not affect tropomyosin in any way, and the protein may best be stored by precipitating from its solution at pH 7 with ethanol, and subsequently drying in several changes of ethanol and then of ether. The protein is precipitated by trichloroacetic acid but redisperses at pH 6·5.

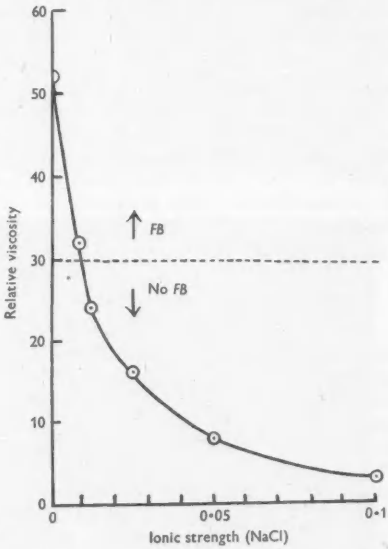


Fig. 2. Relative viscosity of rabbit tropomyosin as a function of ionic strength (Ostwald viscometer). Protein concentration, 0·815%; pH, 6·5; temp. 20°. Arrows indicate presence or absence of flow birefringence (*FB*) at low shear rates.

Viscosity. In salt-free solutions at pH 6·5–7, tropomyosin forms viscous, non-thixotropic solutions which show positive flow birefringence. On addition of neutral salts, there is a dramatic fall of viscosity between the ionic strength values $\mu = 0$ and 0·05, and at the latter value the flow birefringence at low shear rates just disappears. This behaviour is illustrated in Fig. 2, which shows in addition that the lowest values of relative viscosity (e.g. 3·3 when $\mu = 0{\cdot}1$, $c = 0{\cdot}815\%$) are still high in comparison with

those of 'corpuscular' proteins. This high viscosity, even in salt solutions, first suggested that the molecule was inherently asymmetric, a conclusion fully supported by diffusion studies (Bailey *et al.* 1948), X-ray studies (Astbury *et al.* 1948), and by the high values obtained for the intrinsic viscosity. Again, the large increase in viscosity occurring after removal of salt appeared too large to explain except in terms of a polymerization into relatively large fibres, which were later seen in the electron microscope (Astbury *et al.* 1948). This property was difficult to explain until the completion of the amino-acid analysis, when it became clear that it was related, partly at least, to the high complement of dissociating groups which tend greatly to enhance the interaction of the protein with salt and of one protein molecule with another.

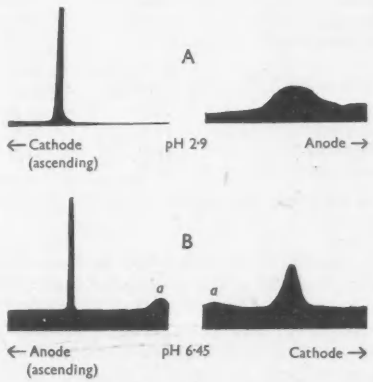


Fig. 3. Electrophoretic patterns of rabbit tropomyosin. (*A*) in 0·071 M-NaCl, 0·02 M-HCl, 0·08 M-glycine (pH 2·9 at 21°); protein concentration, 0·8 %; 4°; overall voltage, 150. (*B*) in 0·05 M-phosphate, pH 6·45; otherwise, as for (*A*) but overall voltage, 200. Boundaries *a*, *a* are stationary and anomalous.

Homogeneity. The protein was submitted to electrophoresis in the Tiselius apparatus at pH 2·9 (glycine buffer) and pH 6·45 (phosphate buffer). The electrophoretic patterns both for the positively and negatively charged protein indicated a complete homogeneity (Fig. 3). Although the ultracentrifuge data obtained by Dr A. G. Ogston (see Bailey *et al.* 1948) likewise indicate a homogeneous substance, the crystals do not conform to a phase-rule solubility. A possible explanation, which may also apply to some other proteins, is that the high water content of the crystals prevents the formation of a stable lattice, which can be so distorted during equilibration of the crystals that ultimately an amorphous product may be produced with a different solubility.

Amino-acid analysis of rabbit tropomyosin

The homogeneous sample of protein which had been submitted to sedimentation and electrophoretic experiments was used for the assay of individual amino-acids. Hydrolysis was effected by refluxing with constant-boiling HCl (10 ml./g. protein) for 24 hr. Excess HCl was removed by repeated evaporation *in vacuo*. In the determination of some components (total N, amide N, P, S, carbohydrate, hexosamine, cysteine + cystine, methionine, tryptophan, tyrosine) hydrolysis was carried out by means specific to the methods cited below.

Methods. Total N (Chibnall, Rees & Williams, 1943) after drying *in vacuo* at 100° over P_2O_5; total P (Allen, 1940); carbohydrate (Pirie, 1936); hexosamine (Palmer, Smyth & Meyer, 1937); total S by alkali fusion (Lugg, 1938); cyst(e)ine and methionine by differential oxidation (Lugg, 1938); tryptophan and tyrosine (Lugg, 1937); amide N by partial hydrolysis at 37° in conc. HCl for 6 days (see Rees, 1946). Basic amino-acids were kindly determined by Dr H. T. Macpherson (Macpherson, 1946); the lysine N, obtained as the difference of catholyte N and histidine plus arginine N agreed well with the value obtained by Dr E. F. Gale employing his manometric decarboxylase method (Gale, 1945). Serine and threonine were determined by Mr M. W. Rees using a modification (Rees, 1946) of the Nicolet & Shinn (1939) method; the figures were corrected for destructive losses during hydrolysis. The monoaminomonocarboxylic acids were assayed by partition chromatography by Dr G. R. Tristram. These values are approximate, since amino-acid recoveries were not checked with the appropriate control mixture (Tristram, 1946). The dicarboxylic amino-acids were quantitatively isolated from 3 g. of protein by the Foreman (1914) lime-ethanol procedure as adapted by Bailey, Chibnall, Rees & Williams (1943). Glutamic acid was independently estimated by Dr E. F. Gale by his decarboxylase method. In the isolation of the dicarboxylic acid derivatives, detailed below, an approximate assay of glycine was obtained from a fraction freed from the bases, dicarboxylic acids and a large part of the less-soluble monoaminomonocarboxylic acids. Two methods were used: (1) the trioxalatochromiate method of Bergmann & Niemann (1937–8); and (2) the chromatographic, now made possible by the absence of the bases and dicarboxylic acids. Both methods revealed so little that the presence of glycine is considered doubtful.

Glutamic and aspartic acids by isolation. The Ca salts were prepared from a sample of hydrolysate containing 0·4683 g. N under the conditions outlined by Bailey *et al.* (1943) for ovalbumin. The initial removal of cyst(e)ine with Cu_2O was omitted since the content of this amino-acid is small. The steps in the analytical procedure may be briefly enumerated and compared with the quantitative data of Table 3. (1) After two lime-ethanol precipitations, the ethanol-soluble amino-acids (*A*) were reserved; the Ca salts (*B*) were decomposed, yielding in the order of isolation, crops GA_1, GA_2 of glutamic acid HCl, AA_1 of Cu aspartate and GA_3. After a third lime-ethanol precipitation of the final mother liquor, the Ca salts yielded GA_4, AA_2, GA_5 and GA_6. Each crop was recrystallized and the mother liquors added to the main filtrate before proceeding to subsequent isolations. (2) The ethanol-soluble salts (*A*) were neutralized to pH 7 with oxalic acid, Ca oxalate removed, and the filtrate evaporated to a small volume *in vacuo*, giving a crystalline monoamino-

acid fraction (*C*). The mother liquor was then treated with Na-free phosphotungstic acid under the conditions recommended by Van Slyke, Hiller & Dillon (1942). The phosphotungstates of the basic amino-acids were recrystallized and discarded, and phosphotungstic acid removed from the combined mother liquors. The final solution of monoamino-acids was freed from inorganic ions: Cl^- by Ag_2O in presence of H_2SO_4; Ag^+ as crystalline sulphate and as Ag_2S; $SO_4^=$ by baryta; Ca^{++} by oxalic acid. The filtrate was evaporated when a further crop of monoamino-acids crystallized. The residual liquor was retained for the determination of glycine.

Table 3. *Dicarboxylic acids and glycine in rabbit tropomyosin*

(Amino-acid N as percentage of total N.)

N in Ca salts (2 precipitations)	29·6
Crops GA_1–GA_6 of glutamic acid HCl	17·6*
(Glutamic acid by decarboxylase method	18·7)
Crops AA_1–AA_2 of Cu aspartate	5·74*
Manipulative losses	0·1
Glycine (trioxalatochromiate method)	0·5
Glycine (chromatographic method)	1·1

Analysis: Glutamic acid HCl. Found: N, 7·64%. Calc.: N, 7·63%. $[\alpha]_D$ +29·4°, indicating admixture with 3·4% of the D-acid. Cu aspartate (water-free basis): Found: N, 7·27; Cu, 32·0. Calc.: N, 7·19; Cu, 32·0%.

* Solubility corrections (Bailey *et al.* 1943) applied.

Analytical characteristics of tropomyosin. In Table 4 are collected the N, P, S and carbohydrate content of tropomyosin and myosin, together with evaluations of those amino-acids determined by two different methods. In Table 5, amino-acid values are converted to g. residues. The source of data for myosin has already been published (Bailey, 1944), but revised values for the basic amino-acids (Macpherson, 1946) are now included. The analysis of myosin is lacking in respect of the dicarboxylic acids, since the titration data of Dubuisson & Hamoir (1943) indicate a higher content than that found by an older isolation method. It must also be remembered that specimens of myosin hitherto analyzed contain a combined adenosine triphosphatase, not yet proved to be an integral part of myosin itself, and some 1–2% actin (Perry, unpublished). With both myosin and tropomyosin, chromatographic methods are approximate but comparative. In the values listed, the second place of decimals is not a guide to the precision of the methods; where results are obtained initially on a weighed quantity of protein, the second place is retained on conversion to a N/N basis (and vice versa) for the sake of averaging.

It will be seen (Table 4) that tropomyosin contains no appreciable amount of P or carbohydrate. Amongst the amino-acids, tryptophan is entirely lacking and glycine probably so.

On comparison with myosin (Table 5) it is at once striking that the amounts of the monoaminomonocarboxylic acids, methionine, tyrosine, alanine, valine, leucines, phenylalanine, serine and the imino-acid proline, are of comparable magnitude. Both proteins are rich in arginine, lysine and glutamic acid, though the amounts vary from one protein to the other. The data in no way suggest that the two proteins are chemically identical, or even that they might be if myosin were freed from probable contaminants. There are large disparities in at least four constituents which are capable of precise assay: threonine, histidine, tryptophan and amide N.

Table 4. *Analysis of rabbit myosin and tropomyosin*

	Tropomyosin (as % protein wt.)	Myosin (as % protein wt.)
Total N	16·6–16·7	16·6 –16·8
P	0·02	0·04– 0·06
Organic S	0·80	1·10
Glucosamine	Nil	Nil
Total carbohydrate	<0·2	<0·2
	(As % protein N)	(As % protein N)
Lysine N (*D*)	17·6	13·6
Lysine N (*E*)	18·0	13·8
Glutamic acid N (*I*)	17·6	>12·6
Glutamic acid N (*E*)	18·7	—

D, N-difference; *E*, enzymic; *I*, isolation methods (see text).

The amount of any one amino-acid has so little significance as yet in the interpretation of structure and biogenetic relationships, that it is most profitable (Astbury, 1942; Bailey, 1944) to compare whole groups of amino-acids expressed as a percentage of the total residues. This gives a precise characterization of the protein in terms of types of side chains. In both myosin and tropomyosin, the amino-acids with non-polar side chains (methionine, tryptophan, phenylalanine, valine, leucines, proline, alanine, glycine) comprise *c.* 35% of the total; the polar side chains are calculated as 63% for tropomyosin and 57% for myosin, the latter figure being minimal on account of analytical deficiencies (see above). The high valence of both proteins is characteristic, and in terms of charges of both negative and positive type, the value of 45% for tropomyosin is higher than in any other protein yet analyzed. The corresponding value for myosin is 34, fibrinogen 26, edestin 27, β-lactoglobulin 28, casein 23, insulin 23 (including end groups), ovalbumin 20 and zein 3%. (It will be noted that the globulins fall in the same group by virtue of their high valence.)

Though this type of comparison again reveals differences between the two proteins, it shows in respect of the most characteristic feature of the myosin molecule, viz. the high content of base and non-amidized carboxyl groups, that tropomyosin is a protein of myosin-type.

Table 5. *Analysis of rabbit myosin and tropomyosin*

(Results calculated on N contents of 16·7 %.)

	Tropomyosin			Myosin		
	N as % protein N	Wt./100 g.	Residues/100 g.	N as % protein N	Wt./100 g.	Residues/100 g.
Cystine/2	0·53	0·76	0·0063	0·98	1·4	0·0117
Methionine	1·58	2·8	0·0188	1·91	3·4	0·0228
Tyrosine	1·44	3·1	0·0172	1·58	3·4	0·0188
Tryptophan	Nil	Nil	—	0·66	0·8	0·0039
Glycine	(0·5)	(0·4)	—	2·1	1·9	0·0253
Alanine	8·3	8·8	0·0988	6·1	6·5	0·0730
Valine	2·25	3·13	0·0267	1·85	2·58	0·0221
Leucines	10·0	15·6	0·1190	10·0	15·6	0·1190
Phenylalanine	2·35	4·6	0·0279	2·2	4·33	0·0262
Proline	0·95	1·3	0·0113	1·4	1·92	0·0167
Serine	3·50	4·38	0·0417	3·46	4·33	0·0412
Threonine	2·04	2·90	0·0244	3·61	5·11	0·0429
Histidine	1·38	0·85	0·0055	3·9	2·41	0·0155
Arginine	15·0	7·8	0·0448	14·2	7·36	0·0423
Lysine	18·0	15·7	0·1074	13·7	11·92	0·0814
Glutamic acid	18·7	32·9	0·2236	12·6	22·1	0·1503
Aspartic acid	5·74	9·1	0·0684	5·6	8·9	0·0669
Amide N	5·35	0·89*	0·0636	7·19	1·20*	0·0857
Totals	**97·11**	**113·72**	**0·8418**	**93·04**	**103·96**	**0·7800**

Average residue wt.:	Tropomyosin	Myosin
(1) From N partition	115·6	115·8
(2) From wt. of residues	117·2	115·3
	Mean 116·4	Mean 115·5
Residues/10^5 g. protein	859	866
As % of total residues:		
Free-acid groups	26·6	18·0†
Base groups	18·4	16·1
	Total 45·0	Total 34·1
Non-polar groups	35·2	35·7
Polar groups	62·8	57·2
Hydroxyl groups	9·7	11·9
Amide groups	7·4	9·9

* Not included in the summations, since the amidized dicarboxylic acids have approximately the same molecular weight as the non-amidized.

† In the calculation for myosin the free-acid groups found by Dubuisson & Hamoir (1943) from titration data (0·156 residues/100 g.) have been used.

DISCUSSION

The present research has attempted a general survey of the properties of tropomyosin rather than a detailed study of any one aspect. Although the main interest must centre upon the role of the protein in the fibril, it should be noted that from the standpoint of its asymmetry and homogeneity, tropomyosin possesses an independent importance as an ideal molecule for the test of hydrodynamical theory.

There is no direct evidence to define the role of tropomyosin, though there are pointers as to its nature. All evidence suggests that it is a prototype of myosin. It is a very elongated molecule (Bailey *et al.* 1948; Astbury *et al.* 1948), capable of electrostatic interaction into relatively enormous fibres of rather regular width and 3000–6000 A. in length. Its solubility properties are similar to those of myosin, particularly in respect of the pH-insolubility zone, its solubility in acid solutions, and the lack of visible denaturation effects when such solutions are neutralized. Like myosin, it possesses a globulin-like solubility, for it not only has a limited solubility in dilute salt solutions on the alkaline side of the isoelectric zone (from pH 6 to 7), but the salting-in effect of neutral salts on tropomyosin crystals is quite marked. Again, the overall characteristics of the amino-acid pattern are of myosin-type, and there are striking similarities in the absolute amounts of many individual amino-acids; more, in fact, than might be expected to arise by mere coincidence. At the same time, there are divergences which refute the idea that myosin and tropomyosin are chemically identical. Lastly, tropomyosin, like myosin, gives a well defined large-angle X-ray pattern of α-type (Astbury *et al.* 1948), and is the first member of the keratin-myosin-epidermis-fibrinogen group to be both genuinely fibrous and genuinely crystalline.

In the light of these properties it is not unreasonable to suggest that tropomyosin may be one of the ultimate units of which myosin is composed (cf. Bailey 1946*b*). *In situ*, it seems to be firmly bound to the substance of the fibril, and requires methods of extraction which suggest that the process is one of metathesis. Its molecular weight of 90,500 (Bailey *et al.* 1948), moreover, is quite comparable with that of myosin after depolymerization in urea solutions, a process which is no longer assumed to involve anything more than the rupture of secondary valence bonds (Mirsky & Pauling, 1936); these subunits of myosin have an average molecular weight *c.* 10^5 (Weber & Stöver, 1933). If in some way the protein is built up into the myosin filament, it might be misleading to cite the spontaneous electrostatic interaction of tropomyosin molecules as being the precise means of aggregation *in vivo*. It demonstrates rather the ability of the molecules to aggregate in regular fashion, whatever the mechanism employed in nature.

Although the hypothesis cannot be enlarged in point of detail, since it concerns an aspect of protein chemistry quite unknown and unexplored, it is a logical extension, however tentative its nature, of the general principles which seem to govern the structure of proteins. In their elaboration there are at least three levels of molecular organization: the first concerns the intramolecular pattern; the second, the aggregation of polypeptide chains (themselves dissimilar in the case of insulin (Sanger, 1945)) into submolecules; and the third, the aggregation of these latter units to give the native protein molecule. If differences exist in the elaboration of a fibrous protein as compared with a corpuscular, they are probably differences of degree rather than of kind, and if it is true that the depolymerization effects observed outside the pH stability zone, or in urea and guanidine solutions, are not due to the cleavage of covalent linkages, then it follows that the stability of the whole molecule is a reflexion only of the number and types of secondary valence forces. Such a generalization appears to be true, at least, of proteins like myosin, in which interchain disulphide bridges are not operative, but even where these do occur, they seem to be utilized in the formation of a stable unit (as in the case of insulin), the aggregation or disaggregation of units being a reversible process involving only secondary valence forces.

A possible, though by no means the only, mechanism (compare, for example, Astbury *et al.* 1948) that can be visualized in the elaboration of a myosin filament is that the necessary cohesion derives from the union of units with specific, interlocking side-chain patterns. Whether we seek the explanation of enzyme action, antigen-antibody formation, or the nature of the unusual colloid interaction of myosin and actin (Bailey & Perry, 1947*a*, *b*), we are led to this conception of specificity as a fundamental property of the protein molecule. It is not illogical, therefore, to invoke the same property as responsible, perhaps, for the elaboration of a native protein from its component units. It is almost inconceivable that a myosin (actomyosin?) filament of some 15,000 A. in length (Hall, Jakus & Schmitt, 1946) could arise fully fashioned from an enzyme template, or that its biogenesis derives from the unfolding of already folded units (cf. Lawrence, Miall, Needham & Shen, 1944). It could be conceived more readily as a process, an autocatalytic process even, requiring only the presence of units with interlocking side-chain patterns. Such units may not be identical, and it is certain, at least, that myosin consists of something more than tropomyosin units.

The experimental verification of the role suggested for tropomyosin is obviously one of great difficulty, but indirectly it would receive strong support if other types of units (cf. Astbury, 1947) from other fibre systems (e.g. the collagens and keratins) are eventually isolated.

SUMMARY

1. A new asymmetric, homogeneous protein, termed *tropomyosin*, has been isolated from skeletal and cardiac muscle.

2. Though water-soluble in neutral solutions after isolation, it can be extracted from the tissue only by salt solutions, and most easily from ethanol- and ether-dried tissue; it thus appears to be firmly attached to the structural components of the fibril.

3. In dilute salt solutions, tropomyosin crystallizes in large birefringent plates containing only 9–11 % of protein. In absence of salt, solutions of the protein are exceedingly viscous, due to the aggregation of molecules by electrostatic interaction into large fibres.

4. In rabbit skeletal muscle it occurs to the extent of 0·5 g./100 g. of wet muscle.

5. The isoelectric point is near pH 5. Its solubility is little affected by heat treatment or by depolymerizing agents such as urea, although the ability to crystallize is lost. On the alkaline side of the isoelectric point, the protein may be dried in organic solvents without loss of solubility.

6. Its amino-acid composition is of myosin type, and like myosin, it gives an X-ray diffraction pattern of α-type.

7. The properties of tropomyosin suggest that it is a prototype of myosin, and the possibility exists that it is one of the units from which the myosin filament is elaborated.

I am much indebted to the various colleagues already mentioned for the application of methods in which they are skilled; to Prof. A. C. Chibnall, F.R.S., Mr G. S. Adair, F.R.S. and Dr E. C. Bate Smith for helpful discussions; to Dr A. G. Ogston for reading the manuscript; lastly and especially to Prof. W. T. Astbury, F.R.S. for his inspiration.

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Molecular Weight of Tropomyosin from Rabbit Muscle

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In the preceding paper (Bailey, 1948) the preparation and properties of tropomyosin are described. The present paper deals with the determination of its molecular weight by three methods: osmotic pressure, sedimentation-diffusion and amino-acid analysis. The partial specific volume and density of the dry protein have also been determined.

METHODS AND RESULTS

Tropomyosin samples. Two preparations have been examined: sample *A*, identical with that used for amino-acid analysis and shown to be electrophoretically homogeneous (Bailey, 1948), and sample *B*, for which there were only analytical criteria for purity.

Osmotic pressure. Measurements were carried out at 0° by the method of Adair (1925) in buffer of composition 0·2 M-KCl, 0·0133 M-Na_2HPO_4, 0·0267 M-NaH_2PO_4, pH 6·5. Protein concentration was determined by the micro-Kjeldahl method, taking 16·7% as the N content of the protein.

Following the procedure of Adair & Robinson (1930), the ratio of osmotic pressure (cm. of water) to protein concentration (P/C) is plotted against C (Fig. 1); concentration is expressed as g./100 ml. solvent, and is obtained by employing the determined partial specific volume (see below) of 0·71. It will be seen that P/C varies considerably with C, whereas for some proteins (e.g. lactoglobulin, ovalbumin, serum albumin) the variation is small over a similar range of concentration, provided that C is expressed as g./100 ml. solvent. This anomaly is probably due either to a thermal interaction between the particles or to a statistical interaction due to their asymmetry. Extrapolation of P/C to zero concentration gives a value of 2·63, indicating a molecular weight of 88,000.

Sedimentation-diffusion data. The sedimentation constant was obtained by examination in the Svedberg oil-turbine ultracentrifuge at Oxford using the method of Philpot (1938). The concentrations of the protein were determined refractometrically, assuming a specific refractive increment of 0·00180; the solvent was as above, but contained NaCl instead of KCl. The speed was 1010 rev./sec.

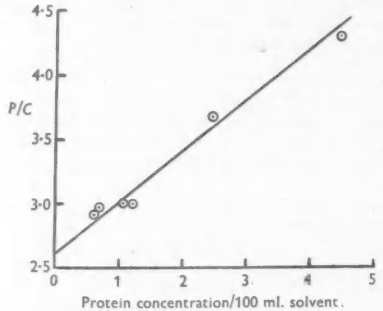

Fig. 1. Osmotic pressure/protein concentration (P/C) as a function of C (rabbit tropomyosin). P in cm. water; C in g./100 ml. of solvent. Buffer: 0·2 M-KCl, 0·0133 M-Na_2HPO_4, 0·0267 M-NaH_2PO_4, pH 6·5, 0°.

The diffusion constant was determined by two different methods. In method 1, measurements were carried out in a cell similar to that of Lamm & Polson (1936) and boundaries were observed by the Philpot (1938) optical system. The diagrams were enlarged photographically and the diffusion constant D calculated from the formula $D=\sigma^2/2t$, where σ represents half the distance between inflexion points, and t the time in sec. The position of inflexion points was located by dividing the maximal height of the curve by $\sqrt{e}$. Two runs were carried out, one at 0·7% protein concentration and one at 1·2%; the solvent was that of the osmotic pressure measurements. Four photographs of the boundary were made during each run, and mean values of D were derived from the four curves of each experiment. Method 2 is essentially new and is described elsewhere (Coulson, Cox, Ogston & Philpot, 1948).

The sedimentation diagram for sample A (Fig. 2) showed a single homogeneous component of s_{20} (corr.) $2{\cdot}60\times10^{-13}$. Integration of the areas of the boundary in the diagram gave 103% recovery of the refractive increment (Philpot, 1939; Johnston & Ogston, 1946), showing that this boundary includes the whole of the sedimenting material. Sample B was likewise homogeneous.

The several values of s and D corrected to 20° and to a water basis are given in Table 1; molecular weights were calculated by the usual formula $M=\dfrac{RTs}{D\,(1-\overline{V}\rho)}$ using a value of 0·71 for the partial specific volume ($\overline{V}$). The mean value from these data is 92,700, somewhat higher than that by osmotic pressure. It should be noted, however, that there is a marked variation of D (and probably also of s) with concentration; strictly, the molecular weight should be estimated from the extrapolated values at zero concentration.

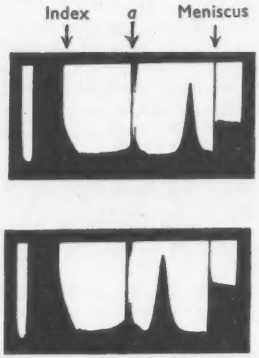

Fig. 2. Sedimentation diagrams of rabbit tropomyosin, 0·6%, 35 and 65 min. after reaching full speed. The shadow marked a is due to aberrant cell washer, which did not interfere with the sedimentation process.

Table 1. *Molecular weight of rabbit tropomyosin from sedimentation-diffusion data in solutions* $\mu=0{\cdot}267$

Sample*	Protein concentration (g./100 ml.)	s_{20} (corr.) $\times10^{13}$	D_{20} (corr.) $\times10^{7}$	Mol. wt.
A	1·20	—	1·78	—
	0·70	—	2·32	—
	0·60	2·60	(2·43)†	89,500
B	0·665	2·51	2·22	94,500
	0·635	(2·55)‡	2·26	94,300
				Mean **92,700**

* D for sample A by method 1; sample B, method 2 (see text).

† By extrapolation of values for 1·2 and 0·7% protein concentration to 0·6%.

‡ By interpolation of values for 0·60 and 0·665% protein concentration.

Molecular weight from the histidine content. Since histidine may be determined accurately (Macpherson, 1946), and since the amount in tropomyosin (0·85 g./100 g. protein) is sufficiently small, the minimal molecular weight multiplied by some small whole number should lead to a reliable value for the true figure. The minimal molecular weight thus obtained (18,180), multiplied by factors of 4, 5 and 6, gives the values 72,700, 90,900 and 109,100 respectively. Of these, only the middle value approaches those found by other methods.

Mean molecular weight. The mean molecular weight, derived from osmotic pressure, sedimentation-diffusion (mean value) and analysis, is 90,500. Using this value and an average diffusion constant of $2{\cdot}35\times10^{-7}$ (probable value at 0·6% protein concentration), D_0, the diffusion constant of a spherical

molecule of similar molecular weight, is found to be $7{\cdot}2 \times 10^{-7}$, giving a frictional ratio $D_0/D = f/f_0 = 3{\cdot}1$. This value, as far as can be ascertained, is much greater than any recorded for a protein of comparable molecular weight, though ideally it should be calculated from the value of D at zero concentration. As in the case of other proteins, the calculation of asymmetry from the frictional ratio is complicated by the uncertain magnitude of the water of hydration. Taken in conjunction with physical and X-ray evidence (Bailey, 1948; Astbury, Reed & Spark, 1948), the high value of f/f_0 indicates in a qualitative manner the pronounced asymmetry of the molecule.

Partial specific volume and density of dry protein. Determinations of partial specific volume ($\bar{V}$) have been made both in water and in salt solutions, using 25 ml. density bottles equilibrated at $20{\cdot}8° \pm 0{\cdot}1$. Corrections were applied for buoyancy and for the small amount of ash in the protein. In salt-free medium the viscosity is so high that it was necessary to evolve a special technique: the isoelectric protein (dried in ethanol, ether and *in vacuo*) was weighed into the bottle and a calculated volume of N/70 NaOH added to give a final pH of 6·5. Within 24 hr. the protein had swollen to a viscous sol from which air bubbles were removed by light centrifuging. Distilled water was now added to capacity and the stopper inserted. Since there was no admixture of protein with the upper water layer, the loss of liquid in this latter operation does not incur loss of protein. A correction was applied for the contribution of Na ions to the density of the medium.

In salt solutions an accurate salt concentration was obtained by adding either NaCl or K_2SO_4 to a dialyzed sol and diluting to 100·0 ml. Samples were then transferred to the density bottle. Protein concentration was determined both by dry weight and by N content, and experiments in which values disagreed by more than 1% were discarded. All samples were measured by weight and not by volume.

The mean value of $\bar{V}$ (Table 2) is 0·71; that calculated by summation of amino-acid residues listed in the previous paper (Bailey, 1948) is 0·735. Because of this discrepancy the details for the determination of $\bar{V}$ have been given at length. It seems clear that the assumption of $\bar{V}$ in the calculation of molecular weights may on occasion give rise to serious error, though it is true that in other cases the calculated values of $\bar{V}$ agree well with the observed (Cohn & Edsall, 1943). It is possible that the discrepancy is confined to proteins with large amounts of acid and base groups, and due either to an intense electrostriction of the molecule itself, or, more probably, to electrostriction of the water of hydration.

Table 2. *Partial specific volume* ($\bar{V}$) *and density* (ρ) *of dry tropomyosin*

Medium	$\bar{V}$	ρ
Water	0·708	—
Water	0·703	—
NaCl (1·0 M)	0·704	—
K_2SO_4 (0·064 M)	0·715	—
Paraffin	—	1·276
Paraffin	—	1·278
Xylene	—	1·280
Mean	**0·71**	**1·28**

The density of tropomyosin after drying at 100° *in vacuo* over P_2O_5 is given in Table 2. In these experiments, measurements were carried out using paraffin or xylene as displacing medium, removing entrapped air bubbles from the protein by evacuating after immersion of protein in the medium. It will be noted that there is a very large discrepancy between the 'apparent density' of the protein in solution ($1/0{\cdot}71 = 1{\cdot}41$) and the determined value for the dry protein (1·28). Similar discrepancies, though not so large, exist in other proteins; the density of dry lactoglobulin (McMeekin & Warner, 1942) is 1·26 and the 'apparent density' from the value of $\bar{V} = 0{\cdot}754$ (Pedersen, 1936) is 1·33.

SUMMARY

1. Tropomyosin from rabbit skeletal muscle is entirely homogeneous in the ultracentrifuge.

2. The molecular weight in salt solutions ($\mu = 0{\cdot}267$) is found to be 88,000 by osmotic pressure and 92,700 by sedimentation-diffusion; the value derived from the histidine content is 90,900 and the mean of all values, 90,500.

3. The osmotic pressure and diffusion constant (and thus probably the sedimentation constant) are markedly dependent upon protein concentration. At the lowest concentration investigated (0·6%), the frictional ratio is 3·1, indicating a very asymmetric molecule.

4. The partial specific volume (0·71) is lower than that calculated from amino-acid residues (0·735).

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An X-ray and Electron Microscope Study of Tropomyosin

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METHODS AND RESULTS

X-rays

A technique that we have found convenient in trying to obtain oriented preparations of elongated macromolecules is to make a thin film by drying a pool of sol on a glass plate, and then to stretch narrow ribbons of the film (Astbury & Dickinson, 1940). The first step tends to leave the molecules lying parallel to the surface of the film but in all azimuths, while the second tends to draw them parallel also to the direction of stretching, so that in the end an X-ray fibre diagram should result. Even without the second step, however, an imperfect fibre diagram may be expected if the specimen is photographed with the X-ray beam parallel to the surface of the film. Further orientation and stretching effects may be produced by squeezing the film between small pieces of plate glass clamped together by screw clips.

Thin films of tropomyosin are readily prepared from the aqueous sol. The X-ray diagrams obtained when the beam is perpendicular and parallel to the film, respectively, are shown in Figs. 1 *a*, *b*. They are typical wide-angle α-patterns of the keratin-myosin-epidermis-fibrinogen group. The fact that Fig. 1 *b* is such a good approximation to a true fibre diagram indicates that the tropomyosin units are so elongated (and probably stiff) that the drying-contraction normal to the surface of the sol forces them to lie down almost flat. The observed spacing of the characteristic meridian arc is 5·11 A., but for the present there is some uncertainty about the side-chain spacing. It is in any case always difficult to measure exactly, but in addition we have noticed an apparent variability at room humidities that, pending further investigation, might reasonably be ascribed to inter-chain swelling by water. The higher estimated values are of the order of 10 A., which is rather greater than what is found for keratin and myosin. The photographs illustrated in Figs. 1 *a* and 2 *a*—they were of an earlier preparation—show also an 'extraneous' halo at about 4·2 A., which is a spacing that we have come to associate with traces of fatty substances, though it does not follow, of course, that that is necessarily the correct interpretation here.

It has not yet been found possible to produce β-tropomyosin by stretching the α-film, either air-dry or over water vapour, for it always breaks after a small percentage extension. In this respect it differs sharply from keratin and myosin. Neither has squeezing at ordinary temperatures been found effective, though it succeeds with keratin, myosin, and fibrinogen (Astbury & Sisson, 1935; Astbury & Dickinson, 1940; Bailey, Astbury & Rudall, 1943). It appears for the moment that heat is necessary: for instance, the disoriented but exceptionally well-developed β-diagram shown in Fig. 2 *a* was obtained by drying a moist specimen at 105°, while Fig. 2 *b* was obtained by squeezing another moist specimen between pieces of plate glass that had first been heated in steam, and then photographing with the X-ray beam parallel to the flat surface. The transformation to β-tropomyosin can take place at much lower temperatures, however, for a good β-diagram was also obtained when the plates were preheated to only 80°.

Fig. 2 *b* is of the less familiar 'cross β' type, i.e. with the backbone reflexion lying on the meridian instead of on the equator; and detailed examination shows that this is due to the fact that hot-squeezing not only transforms the folded α-form into the extended β-form, but also rotates the polypeptide grids so that their side chains stand approximately normal to the plane of flattening. This effect (it indicates a building-up of ribbon-like aggregates of β-grids held together by the backbone linkage) was first observed on squeezing keratin in steam (Astbury & Sisson, 1935), and it was later observed on squeezing moist myosin even at room temperature (Astbury & Dickinson, 1940).

The backbone spacing is again 4·65 A., as found in the other members of the *k-m-e-f* group, but for the present the other two principal spacings, the side-chain spacing and the amino-acid spacing, are not so definite. As in the α-diagram, the former appears to be somewhat higher than usual (it is probably over 10 A. at room humidity), while, in the absence of the normal β-diagram, the latter can only be estimated at 3·2–3·3 A.

Preliminary X-ray examination has been made of tropomyosin crystals in the 'powder' form, air-dry and moist, but nothing has been revealed so far beyond the disoriented α-pattern, plus ammonium sulphate reflexions in the first case and a water halo in the second. It is clear that the α-pattern arises from the structure of the individual tropomyosin units; if the crystals are redissolved and a film is made, the α-pattern is found as before.

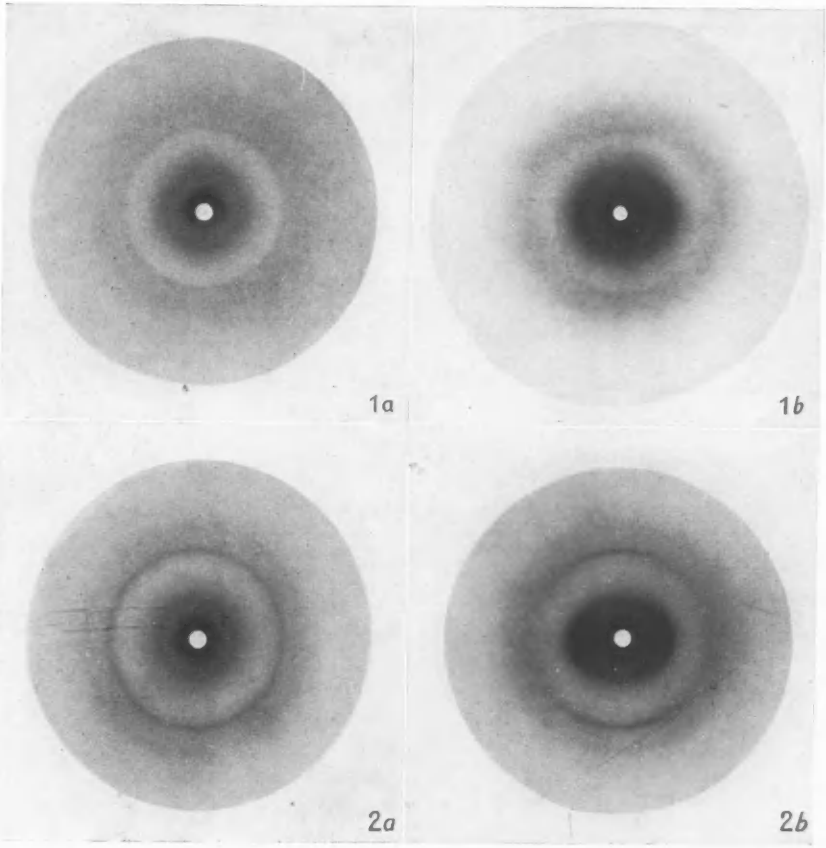


Fig. 1. X-ray photograph of tropomyosin film at ordinary humidity. (*a*) Beam perpendicular to the surface. (Disoriented α.) (*b*) Beam parallel to the surface. (Partially oriented α.)

Fig. 2. (*a*) Disoriented β-diagram obtained by drying moist tropomyosin at 105°. (*b*) Cross β-diagram obtained by squeezing moist tropomyosin between glass plates that had been heated in steam. Beam parallel to the surface.

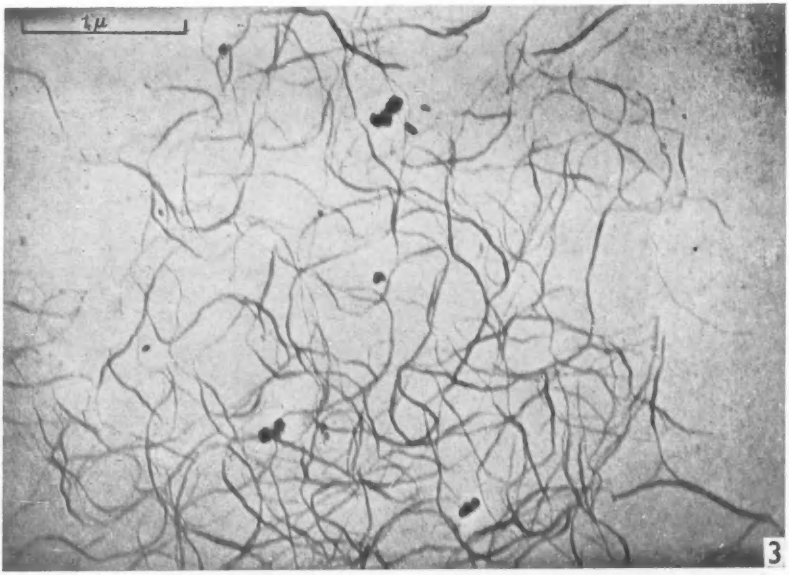


Fig. 3. Tropomyosin fibrils deposited from aqueous solution.

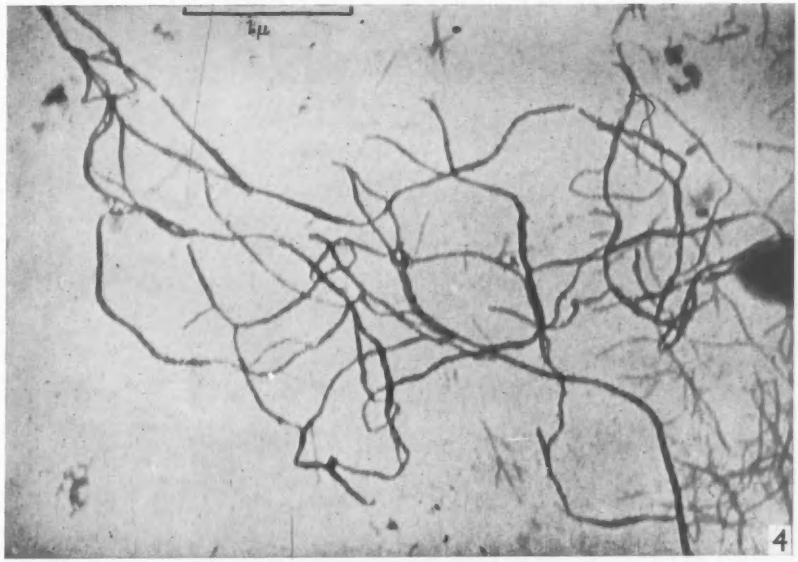


Fig. 4. Tropomyosin fibrils deposited from aqueous solution and stained with osmic acid vapour.

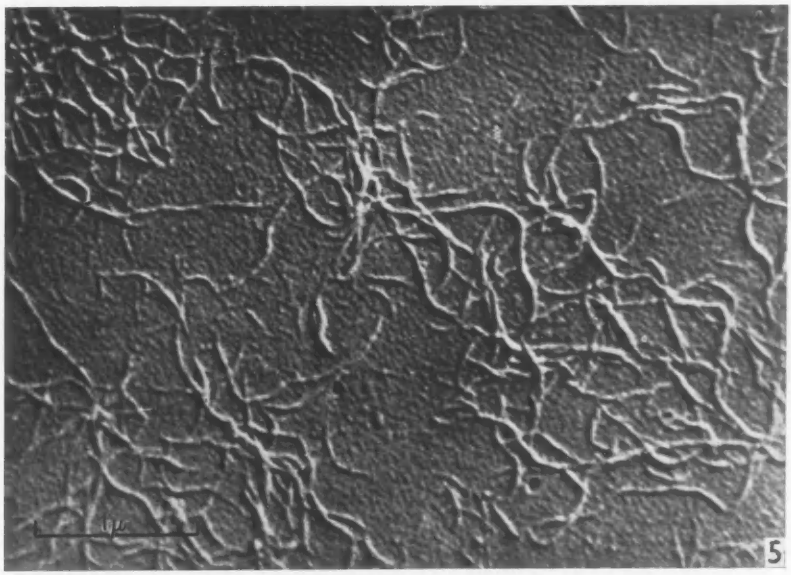


Fig. 5. Tropomyosin fibrils deposited from aqueous solution and shadowed with gold.

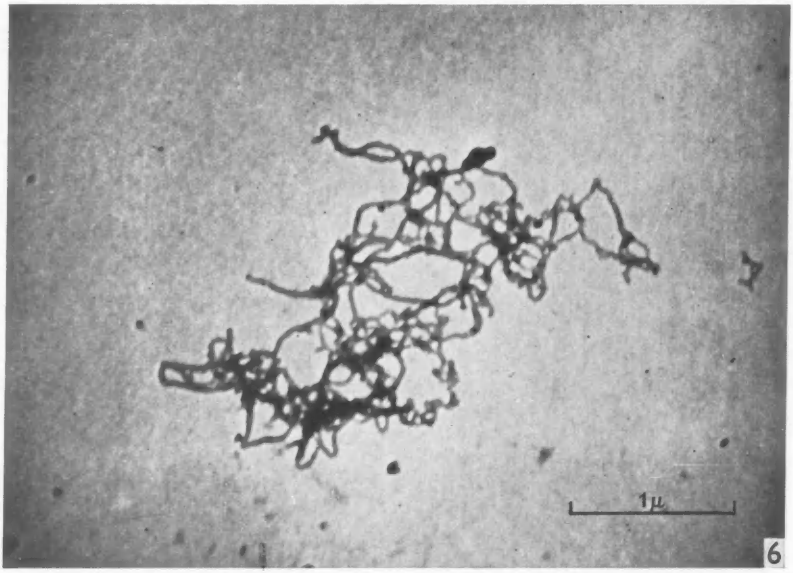


Fig. 6. Tropomyosin fibrils deposited from an aqueous solution that had been heated to 80° for 5 min.

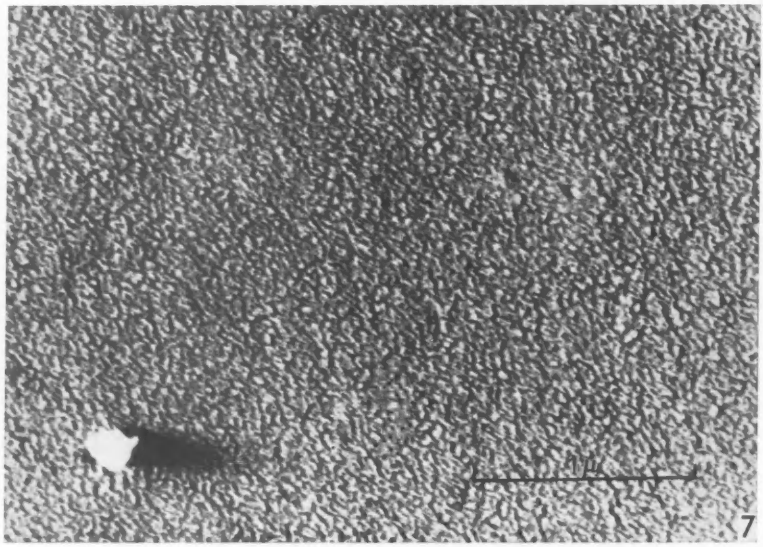


Fig. 7. Deposit from a solution of tropomyosin in 0·1 M-KCl. Gold shadowed.

A few preliminary observations have also been made on the property of 'supercontraction' that tropomyosin might be expected to possess in common with the other members of the *k-m-e-f* group. It was found to be no exception. For this test, short lengths (about 1 cm.) of narrow ribbon cut from a thin film were exposed to steam, and in every case a rapid contraction was recorded, amounting in the mean to some 30% of the initial length. These were admittedly only rough trial experiments, but they leave no doubt that tropomyosin, too, has the power of supercontracting strongly. It may be recalled that similar experiments with myosin films in which the molecules lay approximately flat, but in all azimuths, gave supercontractions of the order of 20% of the initial length (Astbury & Dickinson, 1940).

Electron microscope

The photographs were taken with an RCA instrument, type B. The preparations studied were formed from drops of solution placed on filmed specimen grids and dried by one or other of the following means: air drying at room temperature; accelerated air drying at about 50°; desiccator drying; rapid vacuum drying. The aqueous solutions, at pH 6–7, were made by dissolving in thrice-filtered distilled water the horny solid obtained on drying a concentrated solution, and then diluting to about 1 in 50,000. Two salt solutions were examined, one in 0·1 M-KCl and the other in 0·033 M-KCl, at a concentration of 0·002% protein. For these a drop of solution was placed on the filmed grid and then immediately drained off with filter paper so as to leave a thin layer. The results described here were obtained with tropomyosin prepared from rabbit skeletal muscle, but towards the end of the investigation similar photographs were obtained with fish tropomyosin.

Aqueous solutions were thus shown to give a mesh of very long, fine, and remarkably uniform fibrils, as illustrated in Fig. 3, which is a straightforward micrograph without either staining or metal-shadowing.

Similar dry preparations deposited from aqueous solution were also exposed to the vapour of 1% osmic acid solution, at room temperature, for periods of 1–30 min., and afterwards washed with distilled water. This treatment made the fibrils much bolder in the viewing screen, and in addition it revealed in places a transverse banded, and somewhat saw-tooth, appearance. Fig. 4 illustrates a micrograph of this sort.

The most striking photographs (e.g. Fig. 5) were obtained after shadowing the dry preparations with gold, by the now well-known technique developed by Williams & Wyckoff. The fibrils are seen to be of the order of 200–300 A. thick, and though there were sometimes indications of the banded or beaded appearance suggested by the osmic acid photographs, it was not found possible to confirm this finding outright because of suspicions that the gold may have aggregated. However, it is hoped to repeat these experiments later using chromium.

The action of heat on aqueous solutions of tropomyosin is illustrated by Fig. 6. The solution was heated at 80° for 5 min. and a small drop was quickly transferred to a filmed specimen grid that had been warmed to 50° in a dry tube, after which the preparation was dried as quickly as possible. It will be seen that there is a well-marked change in the character of the fibrils: the network has shrunk and the fibrils have less sharp outlines and have become curled up and often twisted together. Similar experiments at 100° or for longer times produced still more marked effects of this kind.

The action of KCl is illustrated in Fig. 7. The effect is to disperse the fibrils more or less completely; in fact, the 0·1 M solution gave hardly anything on which to focus. After staining with osmic acid vapour a few small fibrils and particles were revealed in the deposit from the 0·03 M solution; but from the 0·1 M solution there were fewer such particles and only poorly resolved micro-fibrils, perhaps about 100 A. thick and a few hundred A. long.

DISCUSSION

The following discussion is based partly on the X-ray and electron microscope results just described and partly on data given in the preceding papers by Bailey (1948) and Bailey, Gutfreund & Ogston (1948).

Tropomyosin is distinguished from other members of the *k-m-e-f* group (except perhaps fibrinogen) in that it consists of relatively short identical chains, or combinations of chains, from which can be prepared both fibres and visible orthodox crystals. It is a 'monomer' of the group (Astbury, 1947*a*, *b*), so to speak, the other members having proceeded presumably to the further stage of end-to-end addition to form much longer chains. It may be a breakdown product of some larger complex already built up in this way, or it may represent 'monomeric' units isolated before 'polymerization', but in either case there can be no doubt of its great importance for the study of the fibrous proteins. It is a key discovery that properly exploited might very well make the decisive contribution towards elucidating the *k-m-e-f* group completely.

Taking the probable value of the molecular weight to be about 90,000, this is equivalent to 90,000/116·4 = 773 residues; and if these residues were incorporated into a single polypeptide chain in the fully extended configuration, their total length would amount to $773 \times 3{\cdot}33 = 2574$ A., and the average side dimensions of the chain would be approximately 10 A. by 4·5 A. The mean axial ratio would thus be well over 300. We have, of course, to consider all possibilities in relation not only to X-ray

data but also to those provided by other techniques, but there is no really convincing argument in favour of this interpretation. Such diffusion and viscosity evidence as is available for the present, though admittedly imperfect, at least points to a considerably smaller axial ratio, and it is hard to believe that such long, flexible chains would build large crystals of the commonplace habit observed. The X-ray photograph given by tropomyosin in its normal state is always an α-diagram, and quite apart from any question of the true nature of the α-configuration, no doubt now remains as to what kind of diagram is given by the fully extended or β-configuration: there is always a strong backbone reflexion of spacing $4{\cdot}6_5$ A., not to mention other characteristic reflexions of weaker intensity. There is nothing in the normal tropomyosin diffraction pattern to suggest either extended chains of the full length quoted above or combinations of similar shorter chains: the diffraction pattern of aggregates of extended chains appears only after treatment to that end.

According to views most favoured at the moment (Astbury & Bell, 1941; Astbury, 1942), the α-form is produced by a regular folding of the backbone (in a plane transverse to the side chains) which reduces the length by very nearly one half. On this interpretation we have, therefore, to consider chains of length about 1287 A., or combinations of submultiple chains which are either distinct or are only loops in the longer chain. Table 1 gives the approximate dimensional characteristics of some of the more

Table 1. *Some possible dimensions of the tropomyosin molecule*

Effective no. of α-chains	Length (A.)	Side dimensions (A.)	Mean axial ratio
1	1287	10 × 9·5	132
2	644	20 × 9·5 or 10 × 19	44
3	429	30 × 9·5 or 10 × 28·5	22

plausible possibilities. These dimensions refer, of course, to the anhydrous molecule, and they are intended to be no more than rough guides for comparison with results that may be obtained by other methods. The alternative side dimensions correspond to the two principal modes of linking parallel polypeptide chains, either by their side chains to form a 'grid' of two or three chains, or by their backbones, in which case they lie on top of one another like centipedes. The first possibility seems the more reasonable, but arguments can be adduced in support of the second.

Table 1, tentative as it is, serves at least to bring out the marked dimensional differences to be expected, and it should not be too difficult to distinguish between the various possibilities by means of critical measurements of diffusion, viscosity, and light scattering. Such measurements are for the present incomplete, but it is informative even now

Table 2. *Calculation of D and ν from possible axial ratios of the tropomyosin molecule*

(See text for explanation of columns.)

(1) Mean axial ratio	(2) D_0/D	(3) D	(4) ν
132	~4·7	$\sim 1{\cdot}54 \times 10^{-7}$	~996
44	2·78	$2{\cdot}60 \times 10^{-7}$	143
22	2·07	$3{\cdot}49 \times 10^{-7}$	45

to make a few comparisons. Table 2 gives estimates of quantities related to diffusion and viscosity. The explanation of the four columns is as follows:

(1) These axial ratios are taken from Table 1. For the purpose of subsequent calculations the assumption is that the molecule approximates to an unhydrated elongated ellipsoid.

(2) D_0 is the diffusion constant of a sphere of the same mass and volume as the ellipsoid. Its radius is given by

$$4/3\pi r^3 \times 1{\cdot}28 = 90{,}000 \times 1{\cdot}65 \times 10^{-24},$$

whence $r = 30{\cdot}3$ A.

Also $$D_0 = \frac{kT}{6\pi\eta r},$$

where k is Boltzmann's constant ($1{\cdot}380 \times 10^{-16}$ erg/degree), T is absolute temperature, and η is the viscosity of the solvent (in this case a buffer at pH 6·5, consisting of 0·2 M-KCl, 0·0133 M-Na_2HPO_4, 0·0267 M-NaH_2PO_4, for which Bailey found a viscosity of approximately 0·0098 poise). Thus D_0 at 20° is found to be $7{\cdot}23 \times 10^{-7}$ cm.²/sec. The ratio D_0/D is equivalent to f/f_0, the 'frictional ratio' of Svedberg & Pedersen (1940), and it is obtained from the axial ratio by aid of a table (Cohn & Edsall, 1943), based on F. Perrin's (1936) equation.

(3) D follows from the ratio D_0/D and the calculated value of D_0.

(4) The quantity ν is the so-called 'viscosity increment' of the solute, and is given by

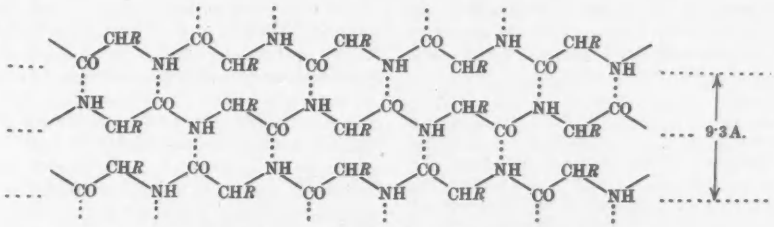
$$\frac{1}{\phi}(\eta/\eta_0 - 1) \equiv \nu,$$

where η is the viscosity of the solution, η_0 that of the solvent, and ϕ is the volume fraction of the system occupied by the solute molecules. It is obtained from the axial ratio by aid of a table based on Simha's equation (Mehl, Oncley & Simha, 1940; Simha, 1940; Cohn & Edsall, 1943).

The available experimental values of D are 2·22 and $2{\cdot}26 \times 10^{-7}$ found by Ogston, and $2{\cdot}43 \times 10^{-7}$ found by Gutfreund on extrapolating to a protein concentration of 0·6 % the values obtained at concentrations of 1·2 and 0·7 %. Bailey (personal communication) has found values of ν equal to 79 and 65 at ionic concentrations of 0·27 and 1·07, respectively. If we assume that axial ratios intermediate between those quoted are ruled out—an

assumption which seems reasonable in the light of all that we know now about the *k-m-e-f* group—then it is fair to say that the diffusion data support tolerably well the double α-chain structure of mean axial ratio about 44. We should expect the observed diffusion constant to be appreciably less than the calculated, principally because we have made no allowance for hydration, which can hardly be neglected with such a polar molecule as tropomyosin, and because, strictly speaking, we need to know the diffusion constant either at infinite dilution or at least for a region of concentration where it does actually remain constant. These omissions represent only the more obvious difficulties: there are, besides, the experimental difficulties associated with the exact measurement of diffusion constants, and in any case the theoretical formulae used are still to be considered as no more than hopeful approximations, pending thoroughgoing test on suitable molecules whose characteristics have been definitely established by independent methods. There would be little justification at this stage in trying to estimate, for example, the degree of hydration required to bridge the gap between prediction and observation, but on the whole it does appear that agreement with a double α-chain is not unsatisfactory. As regards the viscosity data, however, the situation is less encouraging, for the present available values of ν point to something between the axial ratios of 44 and 22; that is, the observed viscosity is *less* than might be expected for a double α-chain. The explanation of this discrepancy may lie in Simha's formula, which is meant to apply only if orientation effects produced by the shearing forces are so small that all molecular orientations may be taken as equally probable. With greatly elongated structures such as we have reason to believe tropomyosin molecules are, the velocity gradients required to meet this condition must be very small indeed, otherwise the recorded viscosity will always be low. At present the experimental data are insufficient to guarantee that Simha's equation would be valid.

The suggestion that a fundamental unit of the *k-m-e-f* group comprises at least two parallel chains (either distinct or forming a loop in a single longer chain) is the more readily acceptable because there are also certain X-ray indications to that effect (Astbury & Woods, 1933). The crystallographic interpretation of the β-diagram requires a unit of pattern which repeats in one of the lateral directions at a distance of *twice* the backbone spacing, and our original explanation of this, which appears to be generally approved, was that it arises from two chains running lengthways but in opposite directions and linked by a succession of CO···NH bridges (now known as the 'backbone linkage') thus:



(The side chains are perpendicular to the plane of the paper and are pointing alternately up and down)

Fibre axis

The electron microscope studies show that the very high viscosity and the easy flow birefringence of aqueous solutions of tropomyosin are consequences of the units aggregating into long, fine fibrils. The latter are observed visually only after evaporation, but there is no doubt that they exist as such in solution so long as salt is absent: when potassium chloride is added the fibrils are broken down again into their units, but the change is reversible on removing the salt. As already mentioned, it is remarkable how uniform the fibrils often are, as though they tend to an almost crystallographic regularity, but this is perhaps not surprising in view of the well-formed and comparatively large crystals that can be grown by other procedures. The micrographs taken after treatment with osmic acid strengthen this impression by their occasional banded appearance, and it may be that some of the micrographs taken after gold-shadowing, though at present open to suspicion, are to be interpreted similarly. By analogy with the yarn-like structure of so many other fibres it would be natural to assume that the long tropomyosin units lie lengthways in the fibrils, overlapping one another irregularly in many regions, but lying strictly abreast or at least overlapping with geometrical regularity in those places where transverse bands are found, but it is not yet possible to verify this assumption directly

by means of the X-ray diagrams because of the difficulty of stretching strips of film without rupture. All that photographs of unstretched film show is that elongated units lie approximately parallel to the surface of the film, but whether parallel or transverse to the fibrils is uncertain; but if a suitable stretching technique could be devised, then it might be possible to orient the fibrils approximately parallel to a single direction and thus reveal which way the units lie *within* the fibrils. It seems very probable that they lie lengthways, otherwise we might expect the flow birefringence to be negative with respect to the direction of flow, whereas it is observed to be positive. Again, the observed usual fibril thickness is of the order of 200–300 A. (and finer fibrils are found too), which is less than the presumed length of even a three-chain unit, let alone the two-chain unit suggested by the diffusion data.

Tropomyosin fibrils present an important example of a phenomenon which has come to the fore only recently, but which promises at last to throw light on the mechanism of formation of protein fibres *in vivo*. It is now well established (Astbury, Dickinson & Bailey, 1935), and indeed has led to successful industrial development (cf. Astbury, 1945), that artificial protein fibres can be made by unfolding and subsequently drawing out the polypeptide chains from the specific configurations of many corpuscular proteins, but whether this process bears any relation to what happens in nature is another question. Actually, the evidence is beginning to point in a direction that is rather an extension of the familiar mechanism of chain formation by polymerization and polycondensation that has been exploited so strikingly in the manufacture of the other great group of man-made fibres, such as nylon and the like, which are truly synthetic. It is for this reason that we have considered tropomyosin as a kind of 'monomer' of the *k-m-e-f* group (Astbury, 1947*a*, *b*): it has the capacity of building up fibres, but as so far observed *in vitro* these are only of an impermanent character; the constituent units are held together by no more than secondary linkages and they can be dispersed again simply by the addition of salt. We can, however, conceive of a further and more permanent step whereby long fibres are built up by the incorporation of covalent linkages between the 'monomeric' units, and it is a 'polymerization' step of this kind that now seems one of the likely final stages in the formation of keratin, myosin and fibrin, and other natural protein fibres. Two other recently recognized examples of the reversible aggregation of corpuscular units to form long, uniform fibrils are fibrous insulin (Waugh, 1946; Hall, 1947) and *F*-actin (Jakus & Hall, 1947; Astbury, Perry, Reed & Spark, 1947). The latter, discovered by Straub (1942, 1943), has been shown by Szent-Györgyi (1942, 1947) and his school to play a critical part in the mechanism of muscular contraction, but its significance in the deeper family relationships of the *k-m-e-f* group may turn out to be more fundamental still (Astbury, 1947*b*, *c*).

The macroscopic crystals of tropomyosin that Bailey has succeeded in growing arouse the greatest interest. Above all they offer an opportunity of single-crystal study by X-ray methods, with the object of discovering ultimately at least the detailed shape if not the actual structure of the units. For the present what has been ascertained is that in the powder form the crystals give the same type of large-angle diffraction pattern as is given by films and by the other members of the *k-m-e-f* group, but it is hoped soon to be able to operate with single crystals. The thoroughly orthodox habit alone is something of a surprise, for it is the sort of thing one associates not with greatly elongated rods but rather with molecules much nearer the spherical; but even more astonishing is the very high water content, amounting to almost 90%. Bailey's figures are: water 89·6, ammonium sulphate 1·74, and protein 8·76%; from which it follows that there are on the average about sixty-six water molecules per amino-acid residue. Another way of describing the situation is to say that on the basis of parallel two-chain units of lateral dimensions approximately 20 × 10 A., each encased in a uniform thickness of water molecules, the mean distance between the surface of one unit and the next comes to about 40 A.! Of course, the longer side chains will project beyond the average, but in the case of the longest (arginine) this will only reduce the distance between the ends of side chains by perhaps 10 A., and we are still left with the impression of a structure that is hardly more than an organized gel, an impression that is confirmed by the great difficulty in even moving the crystals without distorting them.

SUMMARY

1. The large-angle X-ray diffraction diagram of tropomyosin is the characteristic α-diagram of the keratin-myosin-epidermis-fibrinogen group. A similar diagram is obtained whether the specimen is thin film prepared from aqueous solution or a crystal 'powder': presumably, therefore, it arises from the intramolecular pattern of the tropomyosin units themselves.

2. It has not yet been found possible to produce the β-diagram by stretching or squeezing at room temperature, but a well-defined β-diagram appears on heating to above about 80°, and hot-squeezing orients the side chains perpendicular to the plane of flattening, as in other members of the *k-m-e-f* group.

3. Preliminary tests indicate that tropomyosin film shows the property of 'supercontraction' found in other members of the *k-m-e-f* group.

4. In the electron microscope, a deposit from aqueous solution is seen to consist of remarkably uniform fibrils mostly about 200–300 A. thick. These fibrils are completely dispersed into their constituent units by the action of potassium chloride.

5. When stained with osmic acid, and possibly also on shadowing with gold atoms, the fibrils show indications of cross striations.

6. Fibrils deposited from preheated aqueous solution have a shrunken, curled-up and twisted appearance.

7. The structures of the tropomyosin units, fibrils and crystals are discussed in the light of the above findings and data reported in the accompanying communications. It is suggested that tropomyosin is a typical 'monomer' of the *k-m-e-f* group, the long fibres of which are built up by a process of 'polymerization' involving linkages of a more permanent character than those which operate in the formation of the fibrils found in aqueous solution. The latter, however, would appear to be of the nature of impermanent prototypes of the fibres formed *in vivo*.

8. Available diffusion and viscosity data lend reasonable support to the hypothesis that the tropomyosin unit consists of a pair of chains, or a looped single chain, in the α-configuration.

It is clear that the investigation described in this paper could not have been carried out without Dr K. Bailey's close co-operation, and we wish gratefully to acknowledge this and the help of many discussions.

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A Comparison of the Decomposition of Hydrogen Peroxide by Catalase, Ferrous and Ferric Ions, Haemin and Ferrous Phthalocyanine

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The catalytic decomposition of hydrogen peroxide into water and molecular oxygen has been shown to be a very complicated reaction. In the earlier work up to 1923 it was established that the reaction with many different catalysts is quasi-unimolecular: for instance, there are the typical experiments of Bredig & Berneck (1899), Duclaux (1922) and Bertelan (1920) on the decomposition catalyzed by colloidal metals and ferrous and ferric salts. From 1920 to 1932 more detailed kinetic analyses were carried out on the bromine-bromide ion and iodine-iodide ion systems, and the first general kinetic mechanism for the decomposition was devised by Abel (1920) and Bray & Livingston (1923). This is the principle of compensating reactions in which both the oxidized and reduced forms of the catalyst react with the peroxide, giving a dynamic equilibrium between these two states and a resultant catalytic decomposition of the peroxide. For the heavy metal catalysis the chemical mechanism was represented by more or less cumbrous stoicheiometric equations.

The catalytic decomposition of the peroxide is a problem similar to that of the induced reactions studied extensively since the classical experiments of Manchot in 1901. In these reactions there are three components, *A* the actor, *B* the inductor and *C* the acceptor, all oxidizing or reducing agents. In the typical induced reaction *A* can react directly with *B* but not with *C*, also *B* does not react directly with *C*: however, *C* does react with *A* if it is present with the reacting system *A* and *B*. The actor and inductor in many of the systems investigated have been oxygen or hydrogen peroxide and ferrous iron, and to explain the reactions higher oxides of iron, FeO_2, Fe_2O_5 and FeO_3, or iron-peroxide complexes have been suggested (Hale, 1929).

In 1931 Haber & Willstätter suggested that free radicals are intermediates in many chemical reactions in solution, and in 1934 Haber & Weiss published a kinetic study of the decomposition by ferrous and ferric ions and proposed a chain mechanism for the reaction involving $OH^{\cdot}$ and $O_2H^{\cdot}$ radicals, which under certain experimental conditions could become a more simple sequence of radical reactions.

The principle of compensating reactions has been widely accepted for the catalysis of the bromine-bromide ion type, and the Haber & Weiss mechanism has proved an extremely useful working hypothesis for the catalysis by metal ions. It has recently been developed very successfully by Baxendale, Evans & Park (1946) to account for the initiation of the polymerization of certain olefines by ferrous sulphate and hydrogen peroxide in acid solution.

In this and following papers a detailed kinetic analysis of the reaction with various catalysts will be described. There are three features of the decomposition as yet uncorrelated which have directed the experimental work. They derive in turn from the three lines of development of the subject outlined above.

(1) In many of the catalytic systems there is initially a rapid evolution of oxygen, followed by a much slower evolution. This is known as α- and β-activity. It suggests that some steady state is reached in agreement with the principle of compensating reactions.

(2) During this transition from α- to β-activity the equivalent of peroxide destroyed may be in excess of the equivalents of catalyst present. Haber & Weiss (1934) found this for the decomposition with ferrous iron, and it is their chief evidence for the chain character of the reaction. Many oxidase reactions show the same behaviour: Stern has stressed this point in a recent review (1942). The same behaviour occurs in most of the induced reactions. The participation of higher oxides of iron or iron-peroxide complexes was put forward to explain the oxidation of two or three molecules of acceptor whilst the ferrous iron is converted to ferric iron, and side reactions of the higher oxide or complex with hydrogen peroxide, regenerating ferrous iron, can account for greater turnover numbers.

(3) Leaving aside the question of the kinetic mechanism of the reaction, the accepted idea of single electron transfers in this type of oxidation-reduction system makes it necessary to assume the participation of free-radical intermediates in the several unit reactions by which the overall decomposition of the peroxide occurs.

Experiments on the initial evolution of oxygen in the reaction between hydrogen peroxide and catalase, ferrous and ferric ions, haemin and ferrous phthalocyanine are described in this paper, and possible explanations for the transition from α- to β-activity are critically examined.

METHOD

The rate of O_2 evolution has been investigated for reaction mixtures of H_2O_2 and the following catalysts: haemin, ferrous phthalocyanine, ferrous ammonium sulphate, ferric sulphate and liver catalase. The liver catalase was a highly purified preparation obtained from horse liver by the method described by Keilin & Hartree (1945). The kinetic measurements were carried out in two different types of apparatus, Barcroft manometers and a special pressure-gauge apparatus constructed for following the very rapid evolution of O_2 which occurs in the initial stages of the reaction with certain catalysts. Tests were made to ensure that the rate of O_2 evolution measured was a true chemical rate and not conditioned by any physical factors such as diffusion.

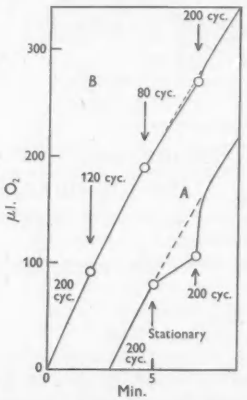

Fig. 1. Diffusion test with Barcroft manometers. Temp. 20°; pH 5·85; oxygen evolved from H_2O_2 + catalase. Shaking speed in cyc./min.

Diffusion test with Barcroft manometers. In these experiments the left-hand flask contained 3·30 ml. H_2O and the right-hand flask 0·30 ml. 0·125 M-phosphate buffer, pH 5·85, 0·10 ml. 2% gelatin solution, 1·10 ml. water and 1·70 ml. H_2O_2 (about 20 vol.) to bring the solution to 1·0 M, together

with the dangling tube holding 0·10 ml. dilute catalase solution. The effect on the reaction rate of keeping the manometers stationary and of varying the shaking speed was determined. The results are given in Fig. 1: from curve *A* it is apparent that physical conditions limit the rate measured when the manometer is stationary, but that on recommencing rapid shaking the true chemical rate is once more established. The latter part of curve *A* is an accurate extrapolation of the former part before shaking was stopped and so the chemical reaction proceeds at the same rate whether the flask is shaken or not. Curve *B* gives similar experimental results on varying the speed of shaking. At shaking speeds above 80 cyc./min. the true chemical rate can be measured: a speed of 150 cyc./min. was used throughout this work.

Diffusion tests with the pressure-gauge apparatus. This apparatus is a simple modification of that described by Meldrum & Roughton (1934) as the 'boat technique'. In

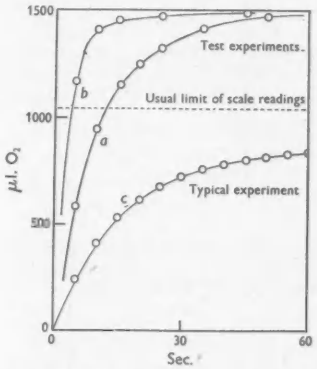

Fig. 2. Diffusion test with pressure-gauge apparatus. Temp. 0°; *a* and *b*, test experiments with $H_2O_2 + KMnO_4$; *c*, typical experiment with $Fe^{++} + H_2O_2$.

place of the oil manometer for measuring the evolution of gas a pressure gauge was used. This consists of a flat brass cylinder (12 cm. diam., 2 cm. deep) soldered at the bottom to a heavy brass plate. A thin brass diaphragm is fitted over the top and held in place by a screwed flange, which enables the tension on the diaphragm to be altered. A fine steel pin (1 cm. high) is fixed to the centre of the diaphragm: the pin is in contact with one end of a small duralumin lever, pivoted about its centre, which carries a galvanometer mirror at the other end. By means of a galvanometer lamp and scale the slight movements of the diaphragm caused by pressure changes in the apparatus are very much magnified. The apparatus is very sensitive to rapid changes of pressure; the lag time is much less than 0·1 sec. The tension on the diaphragm was adjusted, and a test with a subsidiary oil manometer in the system showed that over the range 0–120 scale divisions the deflexion was directly proportional to the pressure change (1 scale division ≡ 0·336 mm. paraffin). The scale can be read to half a division. A set of six boats was calibrated for the apparatus by both the chemical method (acidified $KMnO_4$ and H_2O_2) and the direct method described by Münzer & Neumann (1917). One scale division is approximately equal to 9·2 μl. of O_2. In experiments with this apparatus the scale reading was recorded every 5 sec.

Diffusion tests were carried out at 0° with the same solutions employed in the chemical calibration. The two sides of the boat were filled:

(i) 1·5 ml. 0·0875 N-H_2O_2 + 0·5 ml. H_2O.

(ii) 2·0 ml. 0·195 N-$KMnO_4$ in 0·5 N-H_2SO_4.

The evolution of O_2 from this mixture (1480 μl.) is given in Fig. 2. Curves *a* and *b* show the effect of medium and very fast shaking. The points on curve *b* were independent of any further increase in the shaking speed, so this curve actually represents the chemical rate of the $KMnO_4$-H_2O_2 reaction. The apparatus is clearly capable of measuring true chemical rates of at least 200 μl./sec. The very fast shaking speed was always employed. The results of a typical experiment are shown in curve *c* for the evolution of O_2 from acidified ferrous ammonium sulphate and H_2O_2. The experimental details are:

Boat filled: (i) 0·5 ml. $FeSO_4(NH_4)_2SO_4.6H_2O$ solution (12 g./100 ml.) and 1·5 ml. very dilute H_2SO_4.

(ii) 0·37 ml. H_2O_2 + 1·63 ml. water.

Final concentrations: 0·2 M-H_2O_2, acid ≡ 0·142 ml. N-H_2SO_4.

RESULTS

With some catalysts there is a very rapid evolution of O_2 at the beginning of the reaction which quickly subsides leaving the decomposition proceeding at a much slower rate. The experiments that follow are designed to show whether this effect is to be attributed to the chemical destruction of the catalyst, or whether it is a general kinetic feature of the decomposition arising from the interplay of chemical reactions.

In all the experiments the total volume of O_2 evolved is very small compared with the H_2O_2 concentration present, and so it can be assumed for the kinetic analysis that the peroxide concentration remains constant throughout each experiment. Control experiments with no catalyst present showed the 'blank' reaction to be negligible.

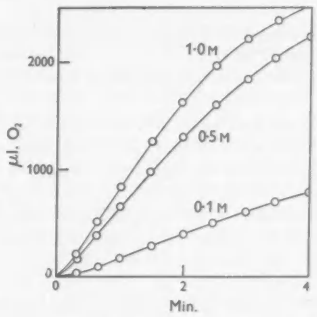

Fig. 3. Haemin. Oxygen evolution with time. Temp. 0°; pH 5·85; identical haemin concentrations. H_2O_2: 1·0, 0·5 and 0·1 M.

Oxygen evolved with haemin. Since haemin is very rapidly destroyed by H_2O_2 at room temperature, these experiments were carried out at 0° with the

pressure-gauge apparatus. The O_2 evolved during the first 4 min. at three different H_2O_2 concentrations is shown in Fig. 3.

The boats were filled:
(i) 0·5 ml. haemin solution (0·117 g. dissolved in a little NaOH and made up to 50 ml.); 0·5 ml. 0·125M-phosphate buffer, pH 5·85, +1·0 ml. water.
(ii) 2 ml. $H_2O_2 + H_2O$ to give required final molarity.

Oxygen evolved with ferrous phthalocyanine. These experiments were carried out in Barcroft manometers at 20°.

The flasks were filled:
Left-hand side, 3·30 ml. H_2O.
Right-hand side, 0·5 ml. 0·125M-phosphate buffer, pH 5·85. H_2O_2 + water to 3·2 ml. giving required molarity. Dangling tube 0·1 ml. iron phthalocyanine suspension containing 0·2 mg. dry weight/ml.

Fig. 4 shows the O_2 evolution at three H_2O_2 concentrations during the first 5 min.

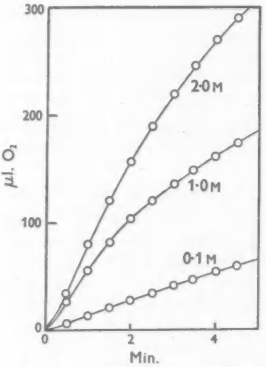

Fig. 4. Ferrous phthalocyanine. Oxygen evolution with time. Temp. 20°; pH 5·85; identical ferrous phthalocyanine concentrations. H_2O_2: 2·0, 1·0 and 0·1 M.

Oxygen evolved with ferrous iron. These experiments were also carried out in Barcroft manometers at 20°. No buffer solution was used: 0·1 ml. of a solution of acidified $FeSO_4(NH_4)_2SO_4 . 6H_2O$ (1·66 g./7 ml.) was placed in the dangling tube, the total acid concentration present being equivalent to 0·565 ml. N-H_2SO_4. The results for 1·0 M and 0·05 M-H_2O_2 are given in Fig. 5.

Oxygen evolved with ferric iron. The decomposition of H_2O_2 in the presence of ferric salts falls off very rapidly as the acidity of the solution is increased. Ferric sulphate in aqueous solution, owing to its hydrolysis, has a very acid reaction, and to obtain a measurable O_2 evolution in these experiments it was necessary to add a very small quantity of NaOH. The amount added was not enough to alter the state of the ferric iron: the solution still had the pale yellow colour characteristic of the ferric iron in acid solution. The experiments were carried out in the pressure-gauge apparatus at 0°. 1·0 ml. of a solution of $Fe_2(SO_4)_3 . 9H_2O$ (8·7 g./50 ml.) + NaOH equivalent to 0·03 ml. N-solution was used in each run, together with H_2O_2 and H_2O to give the required molarity. Typical results are shown in Fig. 6.

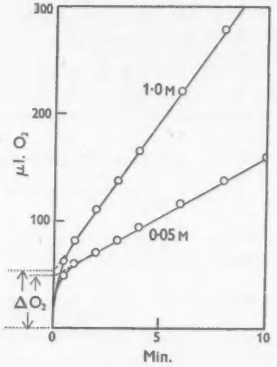

Fig. 5. Ferrous iron. Oxygen evolution with time, showing initial activity. Temp. 20°; acid solution; identical Fe^{++} concentrations. H_2O_2: 1·0 and 0·05 M.

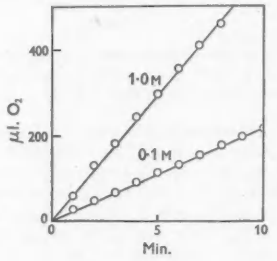

Fig. 6. Ferric iron. Oxygen evolution with time. Temp. 0°; acid solution; identical Fe^{+++} concentrations. H_2O_2: 1·0 and 0·1 M.

Oxygen evolved with liver catalase. Barcroft manometers were used at 20°.

Into the right-hand flask were measured 0·3 ml. 0·125M-phosphate buffer, pH 5·85, +0·1 ml. 2% gelatin solution together with H_2O_2 and water for the required molarity. A very dilute catalase solution (0·1 ml.) was put in the dangling tube. This solution was prepared as follows: stock solution *A* contained 34 mg. catalase/ml. One drop of this solution (0·045 g.) was added to 20 ml. water giving solution *B*. Solution *B* (0·6 ml.) was made up to 50 ml. This was kept in ice in a thermos flask, 0·1 ml. being used for each run.

Typical results for the decomposition with 1·0 M- and 0·2 M-H_2O_2 for the first 15 min. are given in Fig. 7. Fig. 8 shows the results of a similar experiment lasting for 1 hr. Here the catalase concentration was one sixth of its previous strength.

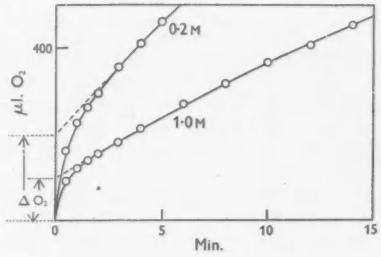

Fig. 7. Liver catalase. Oxygen evolution with time showing initial activity. Temp. 20°; pH 5·85; identical catalase concentrations. H_2O_2: 1·0 and 0·2 M.

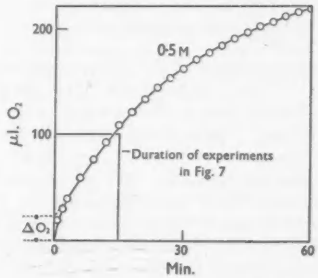

Fig. 8. Catalase. Oxygen evolution with time showing a diminished activity after 15 min., attributed to destruction of the enzyme. Temp. 20°; pH 5·85; 0·5 M-H_2O_2.

DISCUSSION

The destruction of the catalyst

An examination of the experimental curves for the various iron-containing catalysts studied shows that kinetically the catalysts are of two types. Ferrous iron and catalase bring about a reaction in which there is an initial rapid evolution of O_2 which is followed by a much slower steady decomposition. Haemin, ferrous phthalocyanine and ferric iron do not give this initial 'burst' but only the steady rate. The initial evolution in the catalase reaction is extremely interesting: it has been observed by other investigators (Morgulis, Beber & Rabkin, 1926), but as yet no attempt has been made to explain it kinetically and account for it in the mechanism proposed for the reaction. It might be due to the destruction of the enzyme or to some special feature in the decomposition of the peroxide. The observed behaviour with the other catalysts strongly supports the second explanation for the following reasons:

(1) The reaction with catalase and with ferrous iron is very similar (Figs. 5 and 7). With ferrous iron

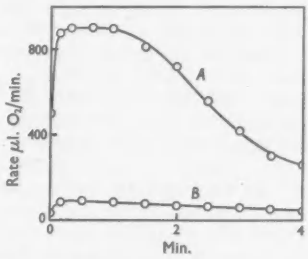

Fig. 9. Variation of rate with time. Values plotted from Figs. 3 and 4. *A*, haemin + 1·0 M-H_2O_2; *B*, ferrous phthalocyanine + 2·0 M-H_2O_2.

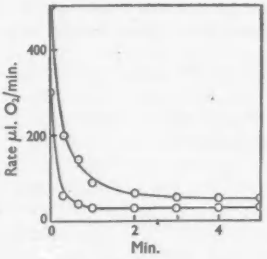

Fig. 10. Variation of rate with time. Values plotted from Figs. 5 and 7. Bottom curve: ferrous iron + 1·0 M-H_2O_2. Top curve: catalase + 0·2 M-H_2O_2.

no destruction of the catalyst can occur, and the kinetic effect here is due to the oxidation of ferrous to ferric iron by the peroxide (Haber & Weiss, 1934; Baxendale *et al.* 1946). There is no initial 'burst' with ferric iron (Fig. 6).

(2) With haemin and ferrous phthalocyanine destruction of the catalyst does occur. The solutions rapidly lose their characteristic colours. This is very marked at high peroxide concentrations (1·0 M and 2·0 M) when the rate falls off after the first minute. At low concentration (0·1 M-H_2O_2) this is hardly noticeable. The initial rapid evolution with catalase and ferrous iron is just as marked at low as at high concentrations. This behaviour can be demonstrated by plotting 'rate' against 'time' as in Figs. 9 and 10: the shape of the curve is completely different in the two cases. With catalase and ferrous iron the rate rapidly falls to an almost constant value (Fig. 10), whereas with haemin and ferrous phthalocyanine the rate continually decreases (Fig. 9).

(3) After the initial activity is over the reaction with catalase proceeds at this almost steady rate, which above a certain peroxide concentration is less the higher the peroxide concentration (Fig. 7). This will be described in detail in a later paper. If enzyme destruction is responsible for this initial activity the rate should diminish continuously to zero. These constant rates at different peroxide concentrations cannot be explained by the enzyme destruction hypothesis.

(4) Enzyme destruction does occur with catalase, but it is only apparent after a much longer time. This is shown in Fig. 8: here the curve from 2 min. to 1 hr. resembles that for haemin from 0 to 5 min.

Thus, whilst the complete destruction of the enzyme cannot be responsible for these kinetic effects, there remains the further possibility that the initial activity results from its partial destruction, leaving a degradation product which is still extremely catalytically active. In a later paper, in which a detailed kinetic study of the reaction will be described, it will be shown that these results cannot be accounted for in this way, and so the initial activity is due to some special feature in the catalytic decomposition of the peroxide.

The following correlation can be made between the state of oxidation of the catalyst and its kinetic effect. The different kinetic behaviour of the various catalysts, the initial rapid evolution of O_2 observed with some and its absence with others, depends on the state of oxidation of the catalyst initially and the concentration of this state in the equilibrium mixture obtained in peroxide solution. In the ferrous-ferric iron system the equilibrium mixture contains predominantly ferric iron, and so if ferrous iron is added to H_2O_2 there is a rapid evolution of O_2 whilst the oxidation of Fe^{++} to Fe^{+++} proceeds. With ferric iron, haemin and ferrous phthalocyanine the initial oxidation state of the catalyst corresponds to that predominating in the equilibrium mixture, and so there is no initial rapid evolution. Catalase, although containing ferric iron, is in the same kinetic class as free ferrous iron. In H_2O_2 solution the initial rapid evolution of O_2 suggests that some type of oxidation-reduction equilibrium is being attained.

The principle of compensating reactions, which provides a qualitative explanation for this, will now be discussed and its quantitative aspects examined. It will be realized that this principle is the general kinetic mechanism for the simple valency change reactions (with the exception of the chain reaction) which have been proposed as mechanisms for the decomposition.

The principle of compensating reactions

The simple picture is that of two reactions; in one the oxidized form of the catalyst is reduced by the peroxide liberating oxygen, in the other the reduced form of the catalyst is oxidized by the peroxide giving water. The net effect of these compensating reactions is the familiar catalytic decomposition:

$$H_2O_2 + 2X \rightarrow O_2 + 2HX$$
$$H_2O_2 + 2HX \rightarrow 2H_2O + 2X$$
$$\overline{2H_2O_2 \rightarrow 2H_2O + O_2}$$

This mechanism has been reviewed by Bray (1932): it has been widely used to explain the catalysis by halogens and the corresponding halide ions. These correspond to the oxidized and reduced state of the catalyst. The mechanism may be illustrated by the decomposition by Br_2 and Br^-.

If H_2O_2 is added to a solution containing Br^- at high H^+ concentration, Br_2 is rapidly formed and soon reaches a constant concentration:

$$H_2O_2 + 2Br^- + 2H^+ \rightarrow Br_2 + 2H_2O. \qquad (a)$$

If H_2O_2 is added to bromine water when either H^+ or Br^- concentrations are low, Br_2 is rapidly reduced to Br^- and oxygen is evolved:

$$H_2O_2 + Br_2 \rightarrow O_2 + 2Br^- + 2H^+. \qquad (b)$$

If the temperature and the final concentrations of H^+ and Br^- are the same as in (*a*) an identical bromine concentration is reached which again remains constant. Under these conditions the rates of the two reactions (*a*) and (*b*) are equal: H_2O_2 is decomposed catalytically—this is the steady state. O_2 is evolved in reaction (*b*), but not in (*a*), so if H_2O_2 is added to bromine water a transition from α- to β-activity will be observed. The reaction with certain heavy metal catalysts is very similar kinetically.

However, for the catalysis by ferrous and ferric ions this simple picture is not sufficient. The obvious compensating reactions in this case are:

$$2Fe^{++} + H_2O_2 \rightarrow 2Fe^{+++} + 2OH^-$$
$$2Fe^{+++} + H_2O_2 \rightarrow 2Fe^{++} + 2H^+ + O_2$$
$$\overline{2H_2O_2 \rightarrow 2H_2O + O_2}$$

Now in the oxidation of Fe^{++} to Fe^{+++} by this mechanism, whilst there would be a rapid destruction of H_2O_2 in attaining the equilibrium state, yet there would be no rapid evolution of O_2, since this arises in the reaction of ferric ion only. This shows conclusively that the reaction is more complicated. The experiments of Haber & Weiss (1934) led them to propose the chain mechanism which can account for it. They measured ΔH_2O_2, the amount of peroxide destroyed in the oxidation of Fe^{++} to Fe^{+++} in neutral solution. Under certain experimental conditions they found that the ratio of ΔH_2O_2 to the initial equivalent concentration of Fe^{++} was greater than one, and attributed this to the length of a chain reaction, initiated by the production of $OH^{\cdot}$ radicals

by Fe^{++} and H_2O_2 and propagated by the reactions of the $OH^{\cdot}$ and $O_2H^{\cdot}$ radicals.

Initiation: $Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^- + OH^{\cdot}$.

Propagation: $OH^{\cdot} + H_2O_2 \rightarrow H_2O + O_2H^{\cdot}$,

$O_2H^{\cdot} + H_2O_2 \rightarrow H_2O + O_2 + OH^{\cdot}$.

The oxygen produced in the second chain propagation reaction explains its evolution in the oxidation of Fe^{++} to Fe^{+++} which could not be accounted for by the simple compensating reactions outlined above. In the gasometric experiments described in this paper the corresponding ratio $\Delta O_2/E_0$, the amount of oxygen evolved in reaching the equilibrium state, to the molar concentration of catalyst initially present can be evaluated. This is important because if the ratio is less than one then the principle of compensating reactions provides an adequate kinetic mechanism: if it is greater than one then either a chain mechanism or another more complex reaction mechanism must be adopted.

In the appendix to this paper the mathematical expression for the ratio $\Delta O_2/E_0$ is derived from the general system of two compensating reactions. If A and B are the two oxidation states of the catalyst, it takes part in two opposing monomolecular reactions which can be represented:

$$A \xrightarrow{k_1} B, \quad A \xleftarrow{k_2} B.$$

The parameters k_1 and k_2 contain peroxide and possibly H^+ ion concentrations together with the reaction velocity constants. When oxygen is evolved in the first reaction and not in the second it is shown that the ratio

$$\frac{\Delta O_2}{E_0} = \frac{k_1^2}{(k_1+k_2)^2}, \quad \text{i.e.} \quad \frac{\Delta O_2}{E_0} < 1.$$

If the compensating reactions are more complicated and oxygen is evolved in both steps then the ratio is still less than one. This ratio can be calculated for the experiments in which ferrous iron and catalase were used.

Ferrous iron (1·0 and 0·05 M-H_2O_2),

$\Delta O_2 = 50\ \mu l. = 2{\cdot}2 \times 10^{-6}$ mol.

$Fe_0^{2+} = 6{\cdot}1 \times 10^{-5}$ mol.

$Fe_0^{2+}/\Delta O_2 = 0{\cdot}037$.

Thus, with the experimental conditions employed, there is no evidence for a chain reaction and a set of compensating reactions is sufficient.

Catalase

$E_0 = 4{\cdot}1 \times 10^{-13}$ mol.

ΔO_2 (1·0 M-H_2O_2) = 100 μl., $\dfrac{\Delta O_2}{E_0} = 1{\cdot}1 \times 10^7$.

ΔO_2 (0·2 M-H_2O_2) = 200 μl., $\dfrac{\Delta O_2}{E_0} = 2{\cdot}2 \times 10^7$.

This is a most striking result, for the ratio is of the order ten million, rather than less than one. It is apparent that the system of two compensating reactions is not an adequate kinetic mechanism. The applicability of the chain mechanism or another more complex mechanism must be examined.

It can be argued from the experiments of Haber & Weiss (1934), on the decomposition by ferrous ions, that this result means that with catalase a chain reaction occurs with a chain length of ten million. However, a comparison of the results with catalase and with ferrous iron presents a serious difficulty. It follows from the mathematical treatment of the compensating reactions given in the appendix that the time for half the initial rapid evolution to occur, $t_{\frac{1}{2}}$, is given by

$$t_{\frac{1}{2}} = \frac{\ln 2}{(k_1+k_2)}.$$

So, from the experimental results, the sum of k_1+k_2 can be evaluated. With 1·0 M-H_2O_2 the values are:

Catalase: $t_{\frac{1}{2}} = 10$ sec., $(k_1+k_2) = 0{\cdot}069$ sec.$^{-1}$.

Ferrous iron: $t_{\frac{1}{2}} = 5$ sec., $(k_1+k_2) = 0{\cdot}139$ sec.$^{-1}$.

The sum k_1+k_2 is of the same order for both ferrous iron and catalase. Now according to the Haber & Weiss (1934) mechanism, where the catalyst both starts and stops reaction chains k_1 and k_2, the parameters involving velocity constants, H_2O_2 and possibly H^+ ion concentrations, refer to the chain-starting and chain-stopping reactions. The measurements of $\Delta O_2/E_0$ suggested that, whilst the ferrous iron reaction does not involve long chains (a sequence of reactions provides an adequate mechanism), the catalase reaction has a chain length of $1{\cdot}1 \times 10^7$ with 1·0 M-H_2O_2. The corresponding chain length for the Fe^{++} ion reaction, 0·037, can be interpreted on the Haber & Weiss mechanism as a more rapid removal of chain centres by reaction with the Fe^{++} ion in the termination process compared with the alternative reaction of the chain centres with H_2O_2 in the propagation reaction. On this basis the ratio of the velocity constants $(k_1^2)_{Fe}$ and $(k_2^1)_E$ for the termination reactions in the Fe^{++} ion and enzyme systems can be estimated. If k_p is the velocity constant for the propagation reaction, then

$$\frac{k_p\,[H_2O_2]}{(k_2^1)_{Fe}\,[Fe^{++}]} = 0{\cdot}037 \quad \text{and} \quad \frac{k_p\,[H_2O_2]}{(k_2^1)_E\,[E]} = 1{\cdot}1 \times 10^7.$$

Substituting the experimental values for H_2O_2, Fe^{++} and catalase concentrations, the ratio $(k_2^1)_{Fe}/(k_2^1)_E$ is found to be 2·0. This implies that the velocity constants of the chain initiation and termination reactions for catalase and ferrous iron are all of the same order. Whilst the H^+ ion concentration is considerably different in the two cases it can hardly account for this odd conclusion. In all comparable

reactions of the iron protoporphyrin protein complexes their velocity constants greatly exceed those with free iron. Thus the simple chain theory does not offer a satisfactory explanation for the action of catalase and the possibility of other explanations should be explored. One alternative mechanism was suggested by Manchot during the course of his work on induced reactions.

Manchot & Lehmann (1928) described experiments with ferrous iron and H_2O_2, the results of which are very similar to those of Haber & Weiss (1934). In the oxidation of Fe^{++} to Fe^{+++} in dilute solution he found that, for each equivalent of Fe^{++} oxidized, three equivalents of peroxide were decomposed, two equivalents of which could react with acceptors. In more concentrated peroxide solutions as many as twentyfour equivalents of peroxide were decomposed for the oxidation of each equivalent of Fe^{++}. He explained the result in dilute solution by the intermediary formation of Fe_2O_5, and in the more concentrated peroxide solutions the increased peroxide decomposition is satisfactorily explained by a reaction between the intermediate and H_2O_2, in which the latter reduces the former back to the ferrous state.

In the absence of more kinetic data it is impossible to reach any definite conclusion regarding the mechanism of the decomposition. Detailed kinetic studies of the reaction with catalase and ferrous iron will be described in future papers.

SUMMARY

1. The evolution of O_2 from H_2O_2 by haemin, ferrous phthalocyanine, ferrous and ferric iron and catalase has been measured manometrically and by the 'boat technique' of Meldrum & Roughton (1934), using a pressure gauge for following rapid rates.

2. There is an initial rapid evolution of O_2 with ferrous iron and catalase but not with the other catalysts. In general, this transition from α- to β-activity might be due to the destructive fission of the catalyst, or to a special kinetic feature of the decomposition with a particular catalyst. Since ferrous iron shows this activity it is not necessarily due to catalyst destruction, for none can occur here.

3. By comparing the catalytic effect of catalase with haemin and iron phthalocyanine it has been shown that complete destruction of the enzyme cannot account for the initial activity, and it is unlikely that partial destruction is responsible.

4. The principle of compensating reactions, which attributes the $\alpha\beta$-activity to the setting up of an equilibrium between two oxidation states of the catalyst, can explain the ferrous iron catalysis but not the reaction with catalase. This follows from the values of $\Delta O_2/E_0$, the ratio of the equivalents of oxygen evolved in the initial rapid reaction to the equivalents of catalyst present, which are 0·037 and $1{\cdot}1 \times 10^7$ respectively for the given experimental conditions.

5. For the catalase reaction the familiar chain mechanism leads to the improbable conclusion that the reaction-velocity constants for chain initiation and termination are each of the same order of magnitude with catalase and ferrous iron.

I am very grateful to Prof. D. Keilin, F.R.S., for his stimulating interest and kind encouragement in this work: and I wish to thank Dr E. F. Hartree for much advice on manometric methods and the preparation of catalase.

Appendix. Compensating Reactions and the Initial Evolution of Oxygen

The relation between the number of equivalents of oxygen evolved in the initial rapid reaction and the equivalents of catalyst present can be obtained in the following way:

$$\underset{(a-x)}{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(b+x)}{B}$$

A and B represent the two oxidation states of the catalyst whose initial concentrations and concentrations at time t are given by a and b, and $(a-x)$ and $(b+x)$ respectively. At time t the rates of conversion of A into B and B into A are given by $k_1(a-x)$ and $k_2(b+x)$, the parameters k_1 and k_2 containing peroxide and possibly hydrogen-ion concentration terms. This is the simplest representation of two compensating reactions; if either involves a sequence of reactions then k_1 and k_2 refer to the rate determining steps in such a sequence.

Moelwyn-Hughes (1940) has shown that in such a system of two compensating monomolecular reactions (monomolecular with respect to the catalyst in the peroxide decomposition)

$$x = \frac{k_1 a - k_2 b}{k_1 + k_2}\left(1 - e^{-\overline{k_1+k_2}\,t}\right).$$

Now if $b=0$, that is the catalyst is present initially in the 'A' state, then the amount of A left at any time is $(a-x)$ where

$$(a-x) = a\,\frac{k_2 + k_1 e^{-\overline{k_1+k_2}\,t}}{k_1+k_2}.$$

Changing to a more familiar nomenclature, if E_0 is the initial concentration of the catalyst (in state A) and E is its concentration at time t,

$$E = E\,\frac{k_2 + k_1 e^{-\overline{k_1+k_2}\,t}}{k_1+k_2}.$$

Now if the k_1 reaction is the one in which oxygen is evolved, the rate at any time R_t is given by

$$R_t = k_1 E = \frac{E_0 k_1 [k_2 + k_1 e^{-\overline{k_1+k_2}\, t}]}{k_1 + k_2}, \qquad (1)$$

and R_0 and R_e, the initial and equilibrium rates when $t=0$ and $t=\infty$ respectively, are

$$R_0 = k_1 E_0, \qquad (2)$$

$$R_e = E_0 \frac{k_1 k_2}{k_1 + k_2}. \qquad (3)$$

The relation between the amount of oxygen evolved in reaching the equilibrium and the mol. of catalyst present is obtained by integrating equation (1):

$$\left.\begin{aligned} R_t = \frac{dO_2}{dt} &= \frac{E_0 k_1 k_2}{k_1 + k_2} + \frac{E_0 k_1^2}{k_1 + k_2} e^{-\overline{k_1+k_2}\, t}, \\ dO_2 &= \frac{E_0 k_1 k_2 dt}{k_1 + k_2} + \frac{E_0 k_1^2}{k_1 + k_2} e^{-\overline{k_1+k_2}\, t}\, dt. \end{aligned}\right\} \qquad (4)$$

Integrating this expression

$$O_2 = \frac{E_0 k_1 k_2 t}{k_1 + k_2} - \frac{E_0 k_1^2}{(k_1 + k_2)^2} e^{-\overline{k_1+k_2}\, t} + I, \qquad (5)$$

where I is the integration constant. This can be evaluated, for when $t=0$, $O_2=0$, therefore

$$I = \frac{E_0 k_1^2}{(k_1 + k_2)^2}.$$

Substituting this value in equation (5):

$$O_2 = \frac{E_0 k_1 k_2 t}{k_1 + k_2} + \frac{E_0 k_1^2}{(k_1 + k_2)^2} (1 - e^{-\overline{k_1+k_2}\, t}). \qquad (6)$$

This is the mathematical expression, according to these simple compensating reactions, for the amount of oxygen evolved at any time. It shows that the experimental curve can be considered in two parts, a straight line through the origin (first term equation (6)) and a curve which rises rapidly to a constant value for O_2 (second term, exponential, equation (6)). This is illustrated in Fig. 11, which is based on Exp. *c* in Fig. 2. Curve A is the experimental curve, B the linear part and C the exponential part: $A = B + C$.

The amount of O_2 evolved in reaching the equilibrium ΔO_2 is given mathematically by the exponential term in equation (6) when $t=\infty$, and

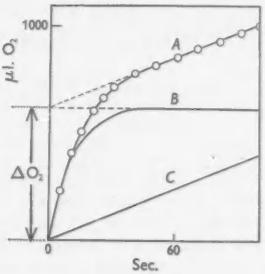

Fig. 11. Analysis of experimental curve (Fig. 2 *c*). Experimental curve, A. Linear portion, C. Exponential portion, B. $A = B + C$.

experimentally by the extrapolation of curve A or C back to the ordinate, i.e.

$$\Delta O_2 = \frac{E_0 k_1^2}{(k_1 + k_2)^2}.$$

Hence the relation between the oxygen evolved and the number of mol. of catalyst present is

$$\frac{\Delta O_2}{E_0} = \left(\frac{k_1}{k_1 + k_2}\right)^2. \qquad (7)$$

If the compensating reactions are slightly more complicated, and oxygen is evolved in both steps, it can be shown that:

$$R_t = \frac{E_0}{k_1 + k_2} [2k_1 k_2 + (k_1^2 - k_1 k_2)\, e^{-\overline{k_1+k_2}\, t}],$$

$$R_0 = k_1 E_0,$$

$$R_s = \frac{2E_0 k_1 k_2}{k_1 + k_2},$$

$$\frac{\Delta O_2}{E_0} = \frac{k_1 (k_1 + k_2)}{(k_1 + k_2)^2}.$$

Thus it is an essential feature of the mechanism based on compensating reactions that the ratio $\Delta O_2/E_0$ should be less than one.

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Studies in Detoxication

18. A STUDY OF THE RELATION BETWEEN CONJUGATION AND DEAMINATION OF *p*-HYDROXYBENZYLAMINE AND RELATED COMPOUNDS IN THE RABBIT

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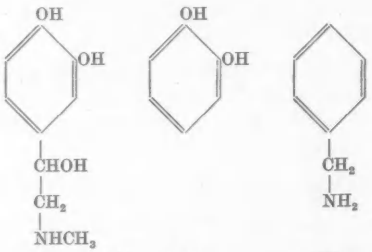
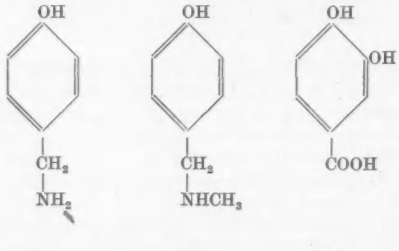
The present work was carried out as part of a programme of research on the inactivation of L-adrenaline (I) in the body. There are two views of the mode of inactivation of adrenaline: briefly, the first is that the inactivation involves monoamine oxidase which oxidatively deaminates adrenaline, and the second is that the inactivation takes place through conjugation of the hydroxyl groups of adrenaline to produce an inactive ethereal sulphate (cf. Richter, 1940; Richter & MacIntosh, 1941–2).

Our plan was to investigate the fate in the body of compounds containing the various structural elements of the adrenaline molecule. Our studies have included, for example, investigations on the metabolism of catechol (II) (Garton & Williams, 1948), *p*-hydroxybenzylamine (IV), *p*-hydroxybenzylmethylamine (V), protocatechuic acid (VI) and D-adrenaline (I) (Dodgson, Garton & Williams, 1947).



(I) (II) (III)



(IV) (V) (VI)

The fate of benzylamine (III) has been studied both *in vivo* and *in vitro*. It is oxidized *in vitro* by amine oxidase preparations from liver and intestinal tissue at about one tenth the rate at which tyramine is oxidized (Blaschko, Richter & Schlossman, 1937), and in the dog it is converted almost quantitatively into hippuric acid (Mosso, 1890), the mechanism of transformation (cf. Green, 1941) being probably as follows:

$$C_6H_5CH_2NH_2 \rightarrow C_6H_5CH{:}NH \rightarrow C_6H_5CHO \rightarrow C_6H_5COOH \rightarrow C_6H_5CONHCH_2COOH.$$

In *p*-hydroxybenzylamine (IV) two changes can take place *in vivo*, namely, conjugation of the OH group and oxidative deamination of the $—CH_2NH_2$ group, and it was our aim to find out how one process influenced the other. We shall show in this paper that conjugation of the OH group is related to the rate of deamination and oxidation of the $—CH_2NH_2$ group.

METHODS

Animals. Chinchilla rabbits, 2–3 kg. wt., on a diet of 50 g. Lever's cubes and water *ad lib.* were used throughout.

Analytical methods. Ethereal sulphate was determined by the Folin gravimetric method (cf. Williams, 1938), glucuronic acid by the method of Hanson, Mills & Williams (1944), and free and combined *p*-hydroxybenzoic acid by the method of Quick (1932) which is based on the bromination method of Day & Taggard (1928).

MATERIALS

A. *p*-Hydroxybenzoic acid, m.p. 213° (all melting points are uncorrected), was prepared by hydrolysis of ethyl *p*-hydroxybenzoate. B. *p*-Hydroxybenzaldehyde, m.p. 115°, was a purified commercial sample. C. *p*-Hydroxybenzylamine was fed as the monohydrate, m.p. 98°, and prepared according to Tiffeneau (1911).

D. *Preparation of* p-*hydroxybenzylacetamide* (N-*acetyl*-p-*hydroxybenzylamine*). This compound appears to be new. *p*-Hydroxybenzylamine (5 g.) was dissolved in a solution of 10 g. K_2CO_3 in 25 ml. water: 10 ml. acetic anhydride were added slowly with shaking, the temperature being maintained below 5°. An oil separated which solidified on standing. The solid was broken up, washed with cold water, dried and recrystallized from benzene. *p-Acetoxybenzylacetamide*, m.p. 62°, forms needles easily soluble in ethanol but insoluble in water (yield, 5 g.). (Found: C, 63·8; H, 6·45; N, 6·7. $C_{11}H_{13}O_2N$ requires C, 63·75; H, 6·3; N, 6·8%.) *p*-Acetoxybenzylacetamide (5 g.) was suspended in a solution of 5 g. KOH in 50 ml. water, and the mixture was refluxed gently until the solid dissolved. Heating was continued until

no oil globules separated on cooling. The solution was now cooled and acidified with HCl. On standing at 0° long white needles of p-*hydroxybenzylacetamide* separated. It was recrystallized from hot water (yield 3·5 g.) and had m.p. 125°. It was slightly soluble in cold water but readily soluble in hot, and it gave a deep violet colour with $FeCl_3$. (Found: C, 65·55; H, 6·8; N, 8·6. $C_9H_{11}O_2N$ requires C, 65·4; H, 6·7; N, 8·5%.)

E. *Preparation of* p-*hydroxybenzylmethylamine.* The method of Tiffeneau (1911) for preparing this amine by heating anisyl chloride with methylamine in a sealed tube was found unsatisfactory owing to the formation of the tertiary di(*p*-hydroxybenzyl)methylamine. We therefore devised a new synthesis.

A solution of 9 g. anisylacetamide (m.p. 97°; Tiffeneau, 1911) in 100 ml. dry benzene was refluxed for 3 hr. with 4 g. finely divided sodamide. The solution, now containing the sodio derivative of anisylacetamide, was cooled and treated with 8 ml. methyl iodide. The mixture was gently refluxed for 10 min., then the NaI which separated was filtered off and the benzene removed by distillation *in vacuo*. The residual *anisylmethylacetamide* was a pale yellow oil (10 g.). This oil was refluxed with 100 ml. N-*n*-butanolic KOH for 2 hr. to remove the acetyl group. The resulting solution was cooled, made strongly acid with conc. HCl and then extracted with 3 × 25 ml. portions of water. The aqueous extract, which contained anisylmethylamine hydrochloride, was made alkaline with solid KOH and extracted with ether. After drying the ethereal solution with solid KOH and filtering, the anisylmethylamine was precipitated as the hydrochloride by passing a stream of dry HCl through the solution. The yield of hydrochloride was 5 g.; it was crystallized from ethanol-ether and had m.p. 165° (Tiffeneau (1911) gives m.p. 166°). Anisylmethylamine was converted into *p*-hydroxybenzylmethylamine hydrochloride (m.p. 185°) according to Tiffeneau (1911).

RESULTS

A. *Experiments with* p-*hydroxybenzoic acid*

(1) *Ethereal sulphate and glucuronic acid outputs.* The figures in Table 1 indicate that about one quarter of the *p*-hydroxybenzoic acid, fed at a dose level of 0·35 g./kg., is excreted conjugated through its hydroxyl group, and that the ratio glucuronic acid/ethereal sulphate is about 2. Our figures are a little higher than those obtained in a more detailed study by Bray, Ryman & Thorpe (1947) for doses of 0·25 g./kg., but our results lead to essentially the same conclusion, viz. that the extent of conjugation of *p*-hydroxybenzoic acid through its hydroxyl group is in the region of 20–30% of the dose.

(2) *Isolation of* p-*hydroxyhippuric acid.* According to Bray *et al.* (1947) about 20–30% of the dose of *p*-hydroxybenzoic acid is conjugated with glycine. We did not repeat this estimation, but isolated the conjugated product.

A total of 6 g. of *p*-hydroxybenzoic acid was fed to 3 rabbits and a 24 hr. urine (350 ml.) collected. The basic lead acetate precipitate of the urine was prepared in the usual manner. This was suspended in water and the Pb removed with H_2S. The filtrate from PbS was continuously extracted with ether for 2 hr. to extract *p*-hydroxybenzoic and *p*-hydroxyhippuric acids. On evaporating the ether a white crystalline residue remained. This residue was extracted with small portions of ether to remove *p*-hydroxybenzoic acid and there remained 300 mg. (3·6% of the dose) of *p*-hydroxyhippuric acid, which after recrystallization from water had m.p. 238° (Bray *et al.* (1947) and Fischer (1908) give m.p. 240°).

(3) *The glucuronide in* p-*hydroxybenzoic acid urine.* Quick (1932) has reported the isolation, from the urine of dogs receiving *p*-hydroxybenzoic acid orally, of *p*-glucuronosidobenzoylglucuronide, a substance which in virtue of its ester glucuronide link reduces alkaline copper reagents. We have been unable to isolate such a glucuronide from rabbit urine and our evidence indicates that, if such a glucuronide is formed, then it must be present in very small amounts (cf. Bray *et al.* 1947).

We found that *p*-hydroxybenzoic acid urines from rabbits were non-reducing, and conclude that the main glucuronide excreted is probably the non-reducing *p*-carboxyphenylglucuronide. We were, however, unable to isolate this substance or a derivative of it in a crystalline state. It was obtained from the basic lead acetate precipitate of *p*-hydroxybenzoic acid urine as a non-reducing yellowish gum. Attempts to form a triacetyl methyl ester did not result in crystalline material. The glucuronide gum (0·5 g.) was hydrolyzed by boiling with 15 ml. of 3–4 N-HCl for 0·5 hr. Ether extraction of the hydrolysate yielded only *p*-hydroxybenzoic acid, m.p. and mixed m.p. 213° (yield of pure material 0·1 g.).

B. *Experiments with* p-*hydroxybenzaldehyde*

(1) *Ethereal sulphate and glucuronic acid outputs.* Table 2 shows that about 40% of the aldehyde is excreted conjugated through its hydroxyl group and

Table 1. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzoic acid in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal value (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal value (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as: Sulphate (%)	Dose excreted as: Glucuronide (%)	Total conjugation (%)
68	2·9	1002	345	19·9	43·8	186·5	239·8	7·7	17·0	24·7
82	2·9	999	344	25·1	47·8	195·3	141·4	8·8	10·1	18·9
84	2·85	1009	253	16·6	66·2	148·6	377·1	12·1	26·6	38·7

the glucuronic acid/ethereal sulphate ratio is about 3·5. The extent of total conjugation is greater than with *p*-hydroxybenzoic acid, the glucuronic acid conjugation of the aldehyde being twice that of the acid, although the sulphate conjugations are the same (cf. Williams, 1938). Since *p*-hydroxybenzaldehyde is largely transformed *in vivo* into *p*-hydroxybenzoic acid (Quick, 1932; Dakin, 1910), it is highly probable, in view of the higher glucuronic reddish orange microcrystalline solid, m.p. 166° (yield 125 mg.). The compound gave positive tests for glucuronic acid, and after acid hydrolysis it gave a red colour for *p*-hydroxybenzaldehyde in the Sammons & Williams (1941*b*) colour reaction. These tests and elementary analysis indicated it to be *p-glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone*. (Found: C, 44·4; H, 4·2; N, 11·6. $C_{19}H_{18}N_4O_{11}.2H_2O$ requires C, 44·4; H, 4·3; N, 10·9%.) A sample of this compound had been prepared by one of us in another investigation and it had m.p. 165° and mixed m.p.

Table 2. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzaldehyde in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal value (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal value (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as: Sulphate (%)	Dose excreted as: Glucuronide (%)	Total conjugation (%)
68	2·75	753	274	14·9	52·7	177·5	391·7	10·7	32·7	43·4
82	2·8	752	269	20·2	45·3	173·8	396·5	9·2	33·1	42·3
84	2·75	751	273	23·9	36·8	197·6	388·1	7·7	32·5	40·2

Table 3. *The ethereal sulphate and glucuronic acid conjugations of* p-*hydroxybenzylamine in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal output (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal output (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as: Ethereal sulphate (%)	Dose excreted as: Glucuronide (%)
91	2·5	624	250	38·1	55·5	—	—	15·7	—
92	2·5	644	258	23·6	80·8	—	—	22·1	—
93	3·0	798	270	37·3	83·9	—	—	18·5	—
99	2·7	663	250	—	—	225·9	406·6	—	44·6
101	2·7	680	252	—	—	221·9	321·8	—	34·4
104	2·5	622	250	—	—	184·0	323·5	—	38·4
							Average	18·8	39·4
							Average total conjugation		58·2

acid excretion provoked by the aldehyde, that some *p*-hydroxybenzaldehyde is conjugated with glucuronic acid before its aldehyde group becomes oxidized. In an earlier paper on vanillin (Sammons & Williams, 1941 *a*) it was shown that the aldehyde glucuronide could be detected in urine in small amounts during the early hours after dosing.

(2) *Isolation of* p-*glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone.* *p*-Hydroxybenzaldehyde (8 g.) was fed to 4 rabbits, and the urine was collected for the following 5 hr. The urine gave a red colour with naphthoresorcinol and HCl in the cold, thus indicating that either *p*-hydroxybenzaldehyde or an *O*-conjugated aldehyde was being excreted (colour reaction of Sammons & Williams, 1941*b*). The urine (300 ml.) was filtered first through cotton wool and then through filter paper, and to it was added 15 ml. of conc. HCl followed by a solution of 1 g. 2:4-dinitrophenylhydrazine in 15 ml. conc. HCl and 100 ml. ethanol. The mixture was kept at 0° overnight, during which time an orange-red precipitate separated. More ethanol was added and the precipitate was filtered and washed with dilute HCl, water and finally ethanol (yield 0·3 g.). The solid was extracted with hot ethanol, and on cooling the extracts there was deposited a with the above sample 166°. (Found: C, 44·5; H, 4·4; N, 11·3%.)

The yield of the hydrazone corresponded to about 2–3% (6% in one case) of the aldehyde fed. None of the conjugated aldehyde could be detected in the urines collected later than 5 hr. after feeding.

C. *Experiments with* p-*hydroxybenzylamine*

(1) *The ethereal sulphate and glucuronic acid conjugations.* The results are given in Table 3 which shows that about 58% of the *p*-hydroxybenzylamine is excreted as *O*-conjugates and that the ratio glucuronic acid/ethereal sulphate is roughly 2. The glucuronic acid conjugation (39%) is slightly higher than that for *p*-hydroxybenzaldehyde (33%), but the ethereal sulphate conjugation (19%) is double that of both *p*-hydroxybenzoic acid and aldehyde (9%). These results could be interpreted as meaning that the amine is partly sulphated before deamination, but conjugated with glucuronic acid after conversion to the aldehyde but before conversion to the acid.

(2) *Isolation of* p-*hydroxybenzoic and* p-*hydroxyhippuric acids.* Three rabbits were each given 2 g. *p*-hydroxybenzylamine with water by stomach tube. The 24 hr. urine (390 ml.) was acidified with 40 ml. of conc. HCl, filtered through glass wool and then extracted with ether continuously for 3 hr. The extract was evaporated to a syrup which crystallized on adding 10–15 ml. of water. The crystals were filtered off, and after two recrystallizations from water yielded white plates of *p*-hydroxybenzoic acid, m.p. 212° and mixed m.p. 213°. The yield after recrystallization was 1 g. or 17% of the dose of amine. This material represents the free acid excreted.

The combined *p*-hydroxybenzoic acid was obtained by continuous ether extraction for 3 hr. of the residual urine, which had been brought to pH 1 and boiled under reflux for 1 hr. The yield of combined acid (m.p. and mixed m.p. 213°) was 1·1 g. or about 18% of the dose of amine.

For the isolation of *p*-hydroxyhippuric acid, a 24 hr. urine, after the feeding of 6 g. of *p*-hydroxybenzylamine, was acidified and extracted with ether as described above. The ether extract was evaporated and the residue extracted with small portions of ether. Most of the residue dissolved, leaving a small residuum of *p*-hydroxyhippuric acid (100 mg., or 1% of the dose of amine). On recrystallization from hot water the acid formed plates, m.p. and mixed m.p. 238°. These isolation experiments account for nearly 40% of the amine fed.

an equal volume of conc. HCl (Sammons & Williams, 1941*b*). The colour increases in intensity in the course of 1 hr. and thereafter it fades to a dirty brown colour. This colour reaction suggests that a conjugate of *p*-hydroxybenzaldehyde is being excreted. An attempt was made, after feeding 8 g. of the amine, to isolate the aldehyde as a 2:4-dinitrophenylhydrazone according to the method already described under the section B(2) on *p*-hydroxybenzaldehyde. A small amount (15 mg.) of a hydrazone was obtained, but we were unable to identify it as *p*-glucuronosidobenzaldehyde-2:4-dinitrophenylhydrazone. The material melted indefinitely, but gave a deep purple colour in dilute NaOH similar to that given by the authentic aldehyde derivative. It also gave the Tollens reaction for glucuronic acid.

In another experiment a 24 hr. urine, after feeding 6 g. of the amine, was collected and the basic lead acetate precipitate was prepared in the usual manner. The Pb was removed with H_2S and the excess H_2S in the filtrate removed by aeration. The filtrate gave a pale red colour in the Sammons & Williams test, but after gentle hydrolysis with HCl a strong red colour was obtained indicating the presence of *p*-hydroxybenzaldehyde. Attempts to prepare an identifiable 2:4-dinitrophenylhydrazone on half of the filtrate failed. The other half was hydrolyzed by boiling for 1 hr. with an equal volume of conc. HCl. The dark solution was cooled, decolorized with charcoal and filtered. This solution gave an intense red colour in the Sammons & Williams

Table 4. *Free and conjugated acid (calculated as* p-*hydroxybenzoic acid) excreted by rabbits receiving* p-*hydroxybenzylamine*

			Acid excreted (calculated as *p*-hydroxybenzoic acid)												Total acid − free acid = conjugated acid (%)
			Blank value on urine before hydrolysis (mg.)	Free acid					Blank value on urine after hydrolysis (mg.)	Total acid					
				Day 1		Day 2		Total		Day 1		Day 2		Total	
Rabbit no.	Wt. (kg.)	Dose of amine (mg.)		(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)		(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	
91	2·5	624	136·5	163	26·7	115	18·8	45·5	118·6	456·3	74·7	114·8	18·8	93·5	48
92	2·5	644	133·1	260·3	41·3	53·3	8·5	49·8	122·6	494·7	78·5	110·8	17·6	96·1	46·3
93	3·0	798	174·1	139·6	17·9	101	12·6	30·5	143·6	498	63·7	119·5	15·3	79·0	48·5

(3) *Quantitative determination of free and combined* p-*hydroxybenzoic acid.* The results are given in Table 4 which show that about 90% of the dose of *p*-hydroxybenzylamine is excreted as *p*-hydroxybenzoic acid. The figures for free *p*-hydroxybenzoic acid include also any *p*-hydroxyhippuric acid, and give, therefore, a measure of the amount of *p*-hydroxybenzylamine excreted as acidic compounds carrying a free phenolic hydroxyl group, i.e. about 40%. The amount of *p*-hydroxybenzylamine excreted as *O*-conjugates is by this method about 47·5%, which is somewhat lower than that found by summation of the glucuronic acid and ethereal sulphate conjugations, 58·1%, given in Table 3. The values obtained by these two methods are, however, of the same order.

(4) *Detection of* p-*hydroxybenzaldehyde derivatives.* The urine collected up to 6 hr. from rabbits after their receipt of doses of *p*-hydroxybenzylamine gives a faint red colour when treated with a little naphthoresorcinol in ethanol and reaction, an intense Tollens reaction and reduced Benedict's reagent. It was extracted with ether to remove any *p*-hydroxybenzaldehyde present. The ether extract was taken to dryness and the residue taken up in 10 ml. ethanol. This ethanolic solution gave a very intense red colour in the Sammons & Williams test, and was treated with 2:4-dinitrophenylhydrazine in ethanol containing H_2SO_4. A small red precipitate, m.p. 270° (after recrystallization from ethanol-benzene), separated. We were unable, however, to identify it definitely as *p*-hydroxybenzaldehyde-2:4-dinitrophenylhydrazone (m.p. 280°) for the quantity obtained was too small for micro-analysis.

We conclude from these experiments that it is very probable that a small amount of conjugated *p*-hydroxybenzaldehyde is excreted after *p*-hydroxybenzylamine has been fed to rabbits, but we have been unable finally to prove this by actual isolation of the aldehyde.

(5) *Detection of nuclear oxidation of* p-*hydroxybenzylamine* in vivo. Bray *et al.* (1947) have shown that *p*-hydroxybenzoic acid is oxidized to a small

extent to protocatechuic acid. They were unable to isolate this acid after feeding *p*-hydroxybenzoic acid, but did so when *p*-hydroxybenzamide was fed. It is therefore possible that, since *p*-hydroxybenzylamine is mainly converted to *p*-hydroxybenzoic acid, the latter may also be slightly oxidized to protocatechuic acid.

p-Hydroxybenzylamine (12 g.) was fed to 6 rabbits and a 24 hr. urine (600 ml.) collected. The urine was acidified with HCl and extracted continuously with ether for 3 hr. Removal of the ether left a crystalline mass, which on recrystallization from water yielded *p*-hydroxybenzoic acid, m.p. 214°. The mother liquors gave no green colour, under the appropriate conditions, with $FeCl_3$, indicating the absence of any free protocatechuic acid. The urine was now made strongly acid with conc. HCl and boiled for 1 hr. It was then cooled and extracted continuously for 3 hr. with ether. Removal of the ether and recrystallization of the residue from water yielded *p*-hydroxybenzoic acid. The mother liquor now gave an intense green colour with $FeCl_3$, which turned blue, violet and finally red on adding $NaHCO_3$. We were unable to isolate crystalline protocatechuic acid from this mother liquor, but there is little doubt that it was present.

We therefore conclude that *p*-hydroxybenzylamine is converted to a very small extent to a catechol derivative which is probably conjugated protocatechuic acid.

(6) *Observations on the glucuronide of* p-*hydroxybenzylamine urine.* The glucuronide of *p*-hydroxybenzylamine urine was obtained via the basic lead acetate precipitate as a gum. Attempts to form crystalline derivatives such as salts or the triacetyl methyl ester were not successful (see section A (3)). The gum was a non-reducing acidic substance, and on acid hydrolysis yielded *p*-hydroxybenzoic acid, m.p. 213°.

We conclude that the glucuronide excreted after feeding *p*-hydroxybenzylamine is largely *p*-carboxyphenylglucuronide.

D. *Experiments with* p-*hydroxybenzylacetamide*

(1) *Ethereal sulphate and glucuronic acid excretion.* Table 5 shows that about 70–80% of *p*-hydroxybenzylacetamide is excreted as *O*-conjugates, the glucuronic acid/ethereal sulphate ratio being about 5.

(2) *The isolation of unchanged* p-*hydroxybenzylacetamide.* A rabbit was fed 2 g. of *p*-hydroxybenzylacetamide and a 24 hr. urine collected. The urine was made acid to Congo red and continuously extracted with ether for 3 hr. The ether was removed and the residue recrystallized (charcoal) from hot water. The crystals (25 mg.) obtained were identified as *p*-hydroxybenzylacetamide, m.p. and mixed m.p. 125°. No trace of *p*-hydroxybenzoic acid was found, and there was no evidence of deacetylation.

(3) *The glucuronide of* p-*hydroxybenzylacetamide.* Each of 3 rabbits was fed 1 g. of the amide and a 6 hr. urine (110 ml.) collected. Very little glucuronide was excreted after the first 6 hr. The basic lead acetate precipitate was prepared in the usual manner and the Pb removed by H_2S. The filtrate from PbS was treated with charcoal, filtered and concentrated to 25 ml. Addition of ethanol to this solution precipitated the glucuronide as an amorphous solid which became gummy on standing. It was therefore redissolved by addition of more water, and the whole was evaporated under reduced pressure to a hard dry amorphous powder (1·5 g.). It could not be induced to crystallize.

The powder was shaken for 10 min. with an ethereal solution of diazomethane (from 1·5 g. of nitrosomethylurea). It formed a gum on the sides of the flask and the mixture was kept at room temperature overnight. During this time the colour of the diazomethane had disappeared, and a further quantity of ethereal diazomethane together with 5 ml. of ethanol were added. The mixture was kept for 48 hr., then decanted from any solid material and taken to dryness at 40°. The residue did not crystallize. It was therefore dissolved in 5 ml. pyridine and 5 ml. acetic anhydride and kept overnight at room temperature. The mixture was then poured into 50 ml. of water and the solution neutralized with solid Na_2CO_3. On standing for 5–6 days, the solution deposited rosettes of fine needles. These were collected and recrystallized from acetone-water (yield 100 mg.). The product, p-*acetamidomethylphenyl triacetylglucuronide methyl ester*, formed rosettes of needles, m.p. 174°, and showed $[\alpha]_D^{20°} - 29{\cdot}05°$ ($c = 1{\cdot}8$ in acetone). (Found: C, 54·95; H, 5·9; N, 3·2. $C_{22}H_{27}O_{11}N$ requires C, 54·9; H, 5·65; N, 2·9%.)

E. *Experiments with* p-*hydroxybenzylmethylamine*

(1) *Ethereal sulphate and glucuronic acid conjugations.* Table 6 shows that about 60% of the amine fed is excreted as *O*-conjugates, the ratio glucuronide/ethereal sulphate being about 8. It is to be noted that the sulphate conjugation is relatively low (7%) and is only a third of that of

Table 5. *The ethereal sulphate and glucuronic acid conjugation of* p-*hydroxybenzylacetamide in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal value (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal value (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as: Sulphate (%)	Dose excreted as: Glucuronide (%)	Total conjugation (%)
99	2·7	610	230	32·2	46·5	202	481	15·7	67·0	82·7
101	2·7	630	233	26·8	21·7	184	657	7·1	88·7	95·8
104	2·5	504	201	35·3	31·7	220	361	13·1	61·0	74·1
110	3·0	795	253	16·3	57·8	153	475	15·7	53·2	68·9
111	3·2	747	234	15·3	51·8	96	465	14·3	52·0	66·3
112	2·9	745	261	15·5	42·2	134	272	11·7	39·1	50·8

p-hydroxybenzylamine (19%). This result is similar to that found for D-adrenaline (Dodgson *et al.* 1947). On the other hand, the glucuronic acid conjugation of this amine (54%) is higher than that of *p*-hydroxybenzylamine (39%), and is approaching that of *p*-hydroxybenzylacetamide (60%).

The main metabolites are free *p*-hydroxybenzoic acid, its ethereal sulphate and ether glucuronide. Small but isolable amounts of *p*-hydroxyhippuric acid and traces of conjugated *p*-hydroxybenzaldehyde and conjugated protocatechuic acid are also excreted.

Table 6. *The ethereal sulphate and glucuronic acid conjugations of p-hydroxybenzylmethylamine hydrochloride in the rabbit*

Rabbit no.	Wt. (kg.)	Dose (mg.)	Dose (mg./kg.)	Ethereal sulphate as SO_3: Mean normal value (mg./day)	Ethereal sulphate as SO_3: Increase after dosing (mg.)	Glucuronic acid: Mean normal value (mg./day)	Glucuronic acid: Increase after dosing (mg.)	Dose excreted as: Sulphate (%)	Dose excreted as: Glucuronide (%)
109	2·5	601	245	28·3	19·7	130	360	7·1	52·4
115	2·6	603	232	27·6	22·2	134	342	8·0	49·6
116	2·6	604	232	19·8	18·9	127	323	6·8	46·7
110	3·0	1474	499	17·5	35·6	137	979	5·2	59·4
111	2·8	1474	526	13·3	54·8	127	1100	7·9	64·1
112	3·2	1500	476	14·2	31·9	62·4	850	4·7	50·5

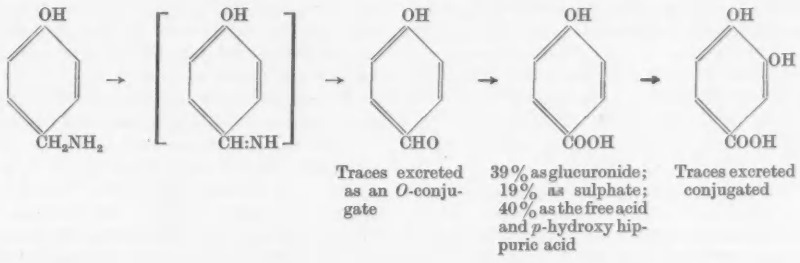
(2) *Isolation of* p-*hydroxybenzoic acid after feeding* p-*hydroxybenzylmethylamine.* Each of 3 rabbits received 1·5 g. of the amine hydrochloride and a 24 hr. urine (1100 ml.) was collected. From 400 ml. of the acidified urine 0·1 g. of *p*-hydroxybenzoic acid, m.p. 214°, was isolated by ether extraction. The extracted urine was strongly acidified with conc. HCl and refluxed for 1 hr. Ether extraction of the hydrolyzed urine yielded 0·1 g. *p*-hydroxybenzoic acid, m.p. 214°.

Thus 45% of the amine fed was accounted for by isolation as *p*-hydroxybenzoic acid, half of the acid occurring in the urine as an *O*-conjugate and the other half free. This experiment indicates that *p*-hydroxybenzylmethylamine is excreted largely as free and conjugated *p*-hydroxybenzoic acid.

DISCUSSION

The ethereal sulphate and glucuronic acid conjugations of the five compounds studied are summarized in Table 7 (p. 302).

The fate of *p*-hydroxybenzylamine in the rabbit can be expressed as follows:



The metabolism of *p*-hydroxybenzylamine involves two separate mechanisms: (*a*) deamination followed by oxidation of the $—CH_2NH_2$ group, and (*b*) conjugation of the phenolic hydroxyl group. It is interesting, therefore, to consider how the one mechanism affects the other. The deamination process can be blocked by acetylating the amino group as in *p*-hydroxybenzylacetamide, and from the figures (see Table 7) for the conjugation of this amide it is clear that when deamination does not take place there is a high *O*-conjugation (73%) particularly with glucuronic acid (60%).

p-Hydroxybenzylmethylamine is intermediate between *p*-hydroxybenzylamine and *p*-hydroxybenzylacetamide in the extent of its conjugation, and this suggests that it is more slowly deaminated than the primary amine. Its rate of deamination is such as to allow a considerable amount of *O*-conjugation to take place. This result indicates that secondary amines of the type $R.CH_2NHCH_3$ are more slowly deaminated *in vivo* than the corresponding primary amines, $R.CH_2NH_2$. This conclusion receives support from the earlier findings of

Ewins & Laidlaw (1910) and of Alles & Heegaard (1943). Ewins & Laidlaw showed that tyramine (*p*-hydroxyphenylethylamine) was readily converted to *p*-hydroxyphenylacetic acid in the perfused dog liver, whereas *p*-hydroxyphenylethyl methylamine formed the acid at a much slower rate. Hordenine (*p*-hydroxyphenylethyldimethylamine) only formed traces of *p*-hydroxyphenylacetic acid after prolonged perfusion. Alles & Heegaard studied *in vitro* the rate of deamination by amine oxidase of derivatives of some sympathomimetic amines, and found the *N*-monomethyl derivatives to be more slowly deaminated than the parent amines. Our results suggest that some idea of the rate of the *in vivo* deamination of phenolic amines may be obtained from a determination of their glucuronic acid conjugations; the possible application of this suggestion to adrenaline is obvious.

Table 7 shows that the glucuronic acid conjugations of the compounds studied form a regular series, but this is not true for the sulphate conjugation. However, sulphate conjugation is complicated by the fact that it occurs in the intestine as well as in the liver (Marenzi, 1931; Arnoldt & De Meio, 1941), whereas the glucuronic acid conjugation occurs only in the liver. It is not possible, therefore, to comment at this stage on the sulphate conjugation of these compounds.

The similarity between the extent of the glucuronic acid conjugation of *p*-hydroxybenzylamine (39%) and of *p*-hydroxybenzaldehyde (33%) suggests that after being fed the amine is largely converted to the aldehyde in the wall of the gut, before being conjugated with glucuronic acid in the liver. Furthermore, this conjugation is virtually complete before the aldehyde is oxidized to *p*-hydroxybenzoic acid, for the glucuronic acid conjugation of this acid (18%) is much lower than that of the aldehyde. The sulphate conjugation of the amine (19%), on the other hand, is much higher than that of the aldehyde (9%), and this may indicate that some sulphate conjugation of the amine occurs in the wall of the gut before it is deaminated to the aldehyde.

Table 7. *The* O*-conjugation of* p*-hydroxybenzylamine and related compounds in the rabbit*

Compound	% of compound excreted as: Ethereal sulphate	% of compound excreted as: Glucuronide	Total *O*-conjugation (% of dose)	Extent of conversion to *p*-hydroxybenzoic acid
HO, $CH_2NHCOCH_3$	13	60	73	Nil
HO, CH_2NHCH_3	7	54	61	Probably complete
HO, CH_2NH_2	19	39	58	Complete
HO, CHO	9	33	42	Complete
HO, COOH	9	18	27	—

SUMMARY

1. The metabolic fates in the rabbit of *p*-hydroxybenzylamine, *p*-hydroxybenzylmethylamine, *p*-hydroxybenzylacetamide, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid have been compared.

2. The main metabolic products of *p*-hydroxybenzylamine are free *p*-hydroxybenzoic acid (*c.* 40%) and the glucuronide (39%) and ethereal sulphate (19%) of *p*-hydroxybenzoic acid. Small amounts of conjugated *p*-hydroxybenzaldehyde, *p*-hydroxyhippuric acid and conjugated protocatechuic acid are also excreted.

3. *p*-Hydroxybenzylmethylamine is also converted into *p*-hydroxybenzoic acid derivatives, but in this case the glucuronide formed is 54% of the dose whereas the sulphate is 7%.

4. *p*-Hydroxybenzylacetamide is not deacetylated *in vivo* and does not produce derivatives of *p*-hydroxybenzoic acid in the urine. It is mainly transformed (60%) into *p*-acetamidomethylphenylglu-

curonide which has been isolated and characterized as its triacetyl methyl ester. *p*-Hydroxybenzylacetamide and its *O*-acetyl derivative have been prepared and described for the first time.

5. *p*-Hydroxybenzaldehyde is largely converted into the glucuronide (33%) and sulphate (9%) of *p*-hydroxybenzoic acid. A small amount (2–3%) is, however, excreted as *p*-glucuronosidobenzaldehyde which has been isolated as a 2:4-dinitrophenylhydrazone.

6. *p*-Hydroxybenzoic acid is the least conjugated of all the compounds studied here and forms only 18% glucuronide and 9% ethereal sulphate.

7. The results indicate that with phenolic amines of the type studied here the extent of glucuronic acid conjugation is inversely proportional to the rate of deamination, the conjugation being highest where no deamination takes place. The significance of this conclusion is discussed.

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Further Observations on the Proteolytic Enzymes in Rat Skin

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The object of this communication is to extend knowledge of the skin proteinase or 'dermoproteinase' previously described by Beloff & Peters (1945) and of its distinction from other proteolytic enzymes present in rat skin (see especially Fruton, 1946).

It was shown that the dermoproteinase will digest casein, serum globulin, serum albumin and myogen, and that it does not fall within the known groups of digestive proteinases by virtue of its failure to digest *N*-benzoyl-L-arginine amide and carbobenzyloxy-L-tyrosylglycine amide, the typical synthetic substrates for trypsin and chymotrypsin; Beloff & Peters (1945) also showed that this proteinase activity could be separated from other peptidases present by differential extraction of the skin.

The experimental results to be presented here will be considered under the separate headings (1) of the effect of methods of extraction upon the enzymes present, (2) of the character of the individual enzymes.

METHODS

Preparation of skin

(*a*) Acetone-dried rat skin was made as described previously (Beloff & Peters, 1945), 0·3 g. of dried skin being equivalent to 1·0 g. of fresh skin. Extracts were prepared so that 5 ml. of extract were equivalent to 0·3 g. of dried skin; either 5% (w/v) aqueous KCl or 5–6% (w/v) aqueous KNO_3 (see below) were used for the extraction of the proteinase.

(*b*) Fresh skin extracts were made by the technique of Fruton (1946). About 30 g. of skin (from two rats), carefully cleaned, were cut into small pieces and stirred with 100 ml. 2% (w/v) NaCl solution in the Waring Blendor for 8–10 min. A further 100 ml. of 2% NaCl were then added, and the whole stirred slowly for 3 hr. at room temperature. The pulpy mass of skin was strained off through muslin, and the extract filtered through a Whatman no. 41 filter paper. Certain comparisons between the extracts obtained by the two methods are given below.

Estimation of activity

The principle was followed throughout of mixing equal volumes of extract and substrate.

(*a*) The peptidase activity was estimated by the titration technique of Linderstrøm-Lang (1927) in 90% (v/v) acetone. That is to say, acetone was added to the aqueous solution to be titrated until the liquid in the titration flask contained 90% acetone, and the titration was then carried out with ethanolic HCl (a standard solution of HCl in acetone being impracticable owing to the volatility of acetone). The synthetic peptide substrates were made up in the required concentration in distilled water, or in veronal buffer of the required pH, according to the extract being used. A 2 hr. digestion period was normally allowed, duplicate 1 ml. samples being taken at the beginning and end of this time. The samples were run into a calculated quantity of 0·1 N-ethanolic HCl, and the titration was completed with 0·01 N-ethanolic HCl. A difference of 0·03 ml. or less (rarely 0·04 ml.) between titration of duplicates was observed.

The synthetic peptides used were supplied commercially by Hoffmann-La Roche and Co. Except in two experiments (Table 5) the racemic, DL peptides were used; a control experiment indicated that the presence of the D peptide exerted no inhibitory effect (see also Beloff & Peters, 1945, addendum).

(*b*) The proteinase activity was estimated by the Van Slyke method. An attempt was made to use the Linderstrøm-Lang titration for this, but it was found unsuitable for the following reasons. Casein, the substrate used, cannot be satisfactorily titrated, since it is precipitated on addition of acetone, the precipitation becoming heavier as the titration proceeds, and being apparently complete at the end point, thus rendering the colour change very difficult to observe. This was assumed to be due to the fact that the pH at the end point (*c.* 4) is approximately the same as at the isoelectric point of casein (4·7). β-Lactoglobulin and serum albumin were satisfactorily titrated, but it was discovered that concentration of salts also affects the accuracy of the titration. Phosphate in particular is precipitated very readily on addition of acetone, giving a rather cloudy effect, and causing a continuous shift of the end point. With 0·1 M-phosphate buffer, titration was not complete for many hours. This effect varies markedly with the salts present, and a solution containing 1% chloride was readily titratable, test-tube experiments indicating that up to 5% chloride would probably be satisfactory. Solutions containing 6% KNO_3 were also satisfactorily titrated. The combination of the effects of protein precipitation and of salts made the titration too uncertain to be useful in this instance.

The presence of 2·5% KCl in the digestion mixture was found to have an unfavourable effect on the Van Slyke method, a grey scum forming in the chamber, and the mercury losing its characteristic physical properties, becoming dull and sticky. This phenomenon was known previously but became in these experiments too troublesome to manage. It was found to be due to a reaction which took place between chloride, nitrite, and mercury in the presence of acid (simplest conditions, HCl, nitrite and mercury). Bromide had a still more marked effect than chloride. The occurrence of the reaction was confirmed using the same amounts of the reagents (A.R.) as were present during our estimation, with a specimen of chemically purified, vacuum-distilled mercury. This led to an attempt to use other salts for the extraction of the skin: a comparatively inactive extract was obtained with sodium acetate and K_2SO_4, whereas 6% (w/v) KNO_3 extracted about two thirds as much activity as 5% KCl. Two experiments are given in Table 1 comparing the extracting activity for dermoproteinase of 5% KCl and 6% KNO_3. In the later experiments 6% KNO_3 was used.

Table 1. *Comparison of* 5% KCl *and* 6% KNO_3 *as extracting agents for dermoproteinase from skin*

(Dried skin (0·3 g.) extracted for 30 min. at room temperature with 5 ml. extraction medium and extract mixed with an equal volume of substrate solution (0·5% casein). Digested at 38° for 2 hr. Proteinase activity estimated by Van Slyke method.)

Exp. no.	Agent	Amino N (mg./100 ml.) Initial	Increase
1	KCl	0·813	0·379
	KNO_3	0·813	0·314
2	KCl	0·861	0·355
	KNO_3	0·888	0·281

Light white soluble casein (British Drug Houses Ltd.) was used; 1% solutions were made up directly in the Waring Blendor with the addition of a little NaOH, the pH being adjusted to the required point with 0·1 N-HCl.

The usual period of digestion of casein solutions and enzyme was 1 hr., unchanged protein being precipitated at the end of that time with 2 ml. 25% (w/v) trichloroacetic acid. The precipitates were washed with 2·5% trichloroacetic acid and the filtrates were made up to 25 ml., 5 ml. samples being estimated.

Control tubes, to which trichloroacetic acid was added immediately after mixing the casein solution and extract, were treated in exactly the same way.

RESULTS

(1) *Effects of methods of extraction upon the enzymes present*

(*a*) *Dried skin extracts.* Extracts of acetone-dried rat skin were previously shown to contain, in addition to the skin proteinase, some peptidase activity for leucylglycylglycine. The peptidase activities were extracted by a lower salt concentration. This was confirmed. Even 0·05 M-phosphate extracted as much or slightly more peptidase than was present in a subsequent extraction of the same skin with addition of 6% nitrate; in contrast to this the higher salt concentration brought out much more proteinase (see Table 2). These extracts also contained some aminoexopeptidase activity, splitting leucylglycine with activation by manganese, though both peptidase activities were weak compared with those of Fruton-type extracts of fresh skin (see Table 3).

Table 2. *Influence of salt concentration upon the extraction of proteases from dried skin*

(Skin extracted as described in Table 1 and extract mixed with equal volume of substrate solution. Digested at 38° for 2 hr. Peptidase activity estimated by Linderstrøm-Lang titration; proteinase activity by Van Slyke method.)

Peptidase activity (substrate, DL-leucylglycylglycine)

Extraction medium	Substrate split (mg./ml.)
1st extraction, 0·05 M-Ringer phosphate	0·17*
2nd extraction, 0·05 M-phosphate + 6 % KNO_3	0·11

Proteinase activity (substrate, 0·5 % casein)

Extraction medium	Amino N (mg./10 ml.) Initial	Increase
1st extraction, 0·05 M-Ringer phosphate	1·671	0·102
2nd extraction, 0·05 M-phosphate + 6 % KNO_3	1·851	0·207

* The concentrations of substrate were not the same in these two experiments, and this figure has been corrected from the curve given (see Fig. 1) to be directly comparable with the other.

It was thought possible that an additional activator, as well as the manganese, was present in the fresh skin extracts, but was partly destroyed by the acetone treatment; this was tested in two experiments. In the first, a normal extract of dried skin was made with 2 % NaCl and portions of this extract were used for a digestion of DL-leucylglycine. To other portions were added small quantities of another very concentrated extract, which had previously been boiled to destroy the enzyme activity, and these were then used for similar digestions. The results were, within limits of experimental error, purely additive, and it was concluded that no heat-stable activator was present.

In the second experiment, samples of DL-leucylglycine were digested (1) with a mixture of the two extracts (dried skin extract and Fruton-type extract), (2) with the same volume of dried skin extract as used in the mixture, and (3) by the same volume of fresh skin extract as used in the mixture. Results are given in Table 4.

The effect, within the limits of experimental error, was again purely additive, and it was concluded that no additional activator was present, the difference between the two extracts being presumably due to the effect of the acetone treatment on the peptidase activity.

Table 3. *Comparison of peptidase activity in different extracts of rat skin*

(Extracts of dried skin made as described in Table 1. Fruton-type extracts made as described under Methods, 1 (*b*). Digestion for 2 hr. at 38°. Estimations by Linderstrøm-Lang titration.)

Extraction medium	Substrate	Substrate concentration (mg./ml.)	pH of digest	Substrate split (mg./ml.) 0·001 M-$MnSO_4$	No activator
		Acetone-dried skin extracts			
2 % NaCl	DL-Leucylglycine	9·4	7·9	0·61	0·26
	DL-Leucylglycine	9·4	7·9	0·33	0·22
	DL-Leucylglycine	9·4	7·9	0·88	0·29
	DL-Leucylglycylglycine	24·5	7·9	0·27	0·19
		Fruton-type extracts			
2 % NaCl	DL-Leucylglycine	9·4	7·9	2·73	0·56
	DL-Leucylglycylglycine	24·5	7·9	3·13	1·52

Table 4. *Test for presence of additional activator in skin*

(Three tubes containing equal amounts of DL-leucylglycine digested for 2 hr. each, (*a*) with a mixture of an acetone-dried skin extract and a fresh (Fruton-type) extract; (*b*) with the dried skin extract alone; and (*c*) with the fresh skin extract alone. The quantity of each extract separately was the same as that present in the mixture of the two, volumes being made up with distilled water.)

Extract	Substrate concentration (mg./ml.)	Leucylglycine split (mg./ml.)
Mixed	9·4	0·87
Fresh skin alone	9·4	0·56
Dried skin alone	9·4	0·35

It is of interest in this connexion to note that Maschmann (1942), working on glycerol extracts of kidney, liver and other tissues, found that desiccation with acetone caused substantial losses in peptidase activity. The enzyme activity most affected was the cleavage of alanylglycylglycine and glycylglycylglycine, while that of leucylglycylglycine and leucylglycine was only slightly altered.

(*b*) *Fresh skin extracts.* Some of Fruton's results were confirmed. The crude extracts contained the aminoexopeptidase and dermopeptidase (see Table 3). The former was found to be present in larger proportion, the latter in lower proportion than found by Fruton in rabbit skin. The two results are compared in Table 5.

Table 5. *Comparison of amounts of peptidase present in rat and rabbit skin*

(The activity of Fruton-type extracts of rat skin compared with Fruton's results on rabbit skin.)

Skin	Substrate	Concentration of L-substrate (mg./ml.)	pH	Time (hr.)	Percentage hydrolysis: No activator	Percentage hydrolysis: 0·001 M-$MnSO_4$
Rat	DL-Leucylglycylglycine	12·25	7·9	2	12·4	25·6
	DL-Leucylglycylglycine	12·25	7·9	2	17·6	30·1
Rabbit (Fruton)	L-Leucylglycylglycine	12·25	7·9	2	24·0	38·0
Rat	L-Leucylglycine	9·4	7·9	2	19·5	78·5
	L-Leucylglycine	9·4	7·9	2	8·4	54·1
Rabbit (Fruton)	L-Leucylglycine	9·4	7·9	5	3·0	4·0

The proportionately greater effect of manganese in the case of the leucylglycylglycine-splitting activity of rat skin, as opposed to rabbit skin, is presumably due to the greater proportion of the leucine aminopeptidase present.

It was confirmed that dialysis had the effect, reported by Fruton, of removing the leucine aminopeptidase activity, leaving the leucylglycylglycine-splitting activity which is not activated by manganese, though, in the present work, a longer period of dialysis was necessary. The dermopeptidase activity against leucylglycylglycine was also reduced, apparently to an extent greater than proportional to the dilution occurring during dialysis. A volume of 26 ml. of the crude extract was dialyzed for 3 days (Fruton's time) at about 1° against 1500 ml. of distilled water: 29 ml. of extract were collected from this dialysis, and it was then found that the leucylglycylglycine-splitting activity had been reduced by half, the leucylglycine-splitting activity by only one third. The remaining extract was dialyzed against 4 l. distilled water for a further 12 hr., during which time no further change in volume occurred. It was then found that the leucylglycylglycine-splitting activity had remained constant, while the leucylglycine-splitting activity had fallen markedly, though it was still not entirely removed. The results are given in Table 6.

The fresh skin extract was found to contain the dermoproteinase, though the amount was low compared with that in the dried skin extracts. Hence this enzyme is again proved to be distinct from the other two. Results are given in Table 7.

Table 6. *Effect of dialysis upon the peptidases of rat skin*

(Fruton-type extract dialyzed twice, once for 3 days, then again for 12 hr. (see text). Activities against leucylglycine and leucylglycylglycine are given after each dialysis for comparison with original values.)

Dialyzed	Substrate	Substrate concentration (mg./ml.)	pH	Time (hr.)	Substrate split (mg./ml.): No activator	Substrate split (mg./ml.): 0·001 M-$MnSO_4$
Not dialyzed	DL-Leucylglycine	9·4	7·9	2	0·56	2·73
Once	DL-Leucylglycine	9·4	7·9	2	0·38	—
Twice	DL-Leucylglycine	9·4	7·9	2	0·11	0·30
Not dialyzed	DL-Leucylglycylglycine	24·5	7·9	2	1·52	3·13
Once	DL-Leucylglycylglycine	24·5	7·9	2	0·74	—
Twice	DL-Leucylglycylglycine	24·5	7·9	2	0·76	0·76

Table 7. *Dermoproteinase activity of Fruton-type extracts of fresh rat skin compared with that of dried skin extracts*

(Extracts (5 ml.) made as previously described, mixed in each case with 5 ml. of a 1% casein solution, and digestion allowed to proceed at 38° for 1 hr. Amino N determined by Van Slyke method.)

Extract	Skin preparation	Amino N (mg./10 ml.): Before digestion	After digestion	Increase
Dried skin	—	—	—	0·329*
Fresh skin	1	0·844	0·927	0·083
Fresh skin	2	0·970	1·181	0·211
Fresh skin	3	0·463	0·603	0·139
Fresh skin	4	0·523	—	0·154
Extract of residual skin	4	0·262	—	0·254†

* Average of ten determinations. † One determination.

In addition, the skin remaining after the Fruton-type extract had been made was re-extracted with 6% KNO_3 in the proportion 1 g. fresh skin (≡0·3 g. dried skin) to 5 ml. 6% KNO_3 and the peptidase and proteinase activities compared with the original extract. Tables 7 and 8 show that there was little peptidase, but good proteinase activity in the final KNO_3 extract.

In one experiment using DL-leucylglycine as substrate (9·4 mg./ml.) at pH 7·9 and a digestion period of 2 hr., an extract of rat skin gave a splitting of 1·12 mg./ml. without addition, which was increased to 1·5 mg./ml. by addition of 0·01 M-$MgSO_4$ and to 2·17 mg./ml. by 0·002 M-$CoCl_2$. Berger & Johnson (1939) stated that the leucylpeptidase activities of their preparations were unaffected and slightly inhibited by cobalt, whereas Maschmann (1940) found that the leucylpeptidase activities of sera were activated by cobalt though less so than by manganese, a result consistent with our experiment.

Table 8. *Comparison of peptidase activities of Fruton-type extract of rat skin, and of extract made of residual skin with* 6% KNO_3

(Extracts made as described in Table 1.)

Extract	Substrate	Concentration of L-substrate (mg./ml.)	pH	Time (hr.)	Percentage hydrolysis: No activator	Percentage hydrolysis: 0·001 M-$MnSO_4$
Fruton-type	DL-Leucylglycylglycine	12·25	7·9	2	17·6	30·1
Fruton-type	L-Leucylglycine	9·4	7·9	2	8·4	54·1
Residual skin	DL-Leucylglycylglycine	12·25	7·9	2	3·4	3·6
Residual skin	DL-Leucylglycine	9·4	7·9	2	2·5	3·1

(2) *Some further details about the enzymes*

Substrates. Precise data about the optimal concentration of substrates could not be found in the literature: the results of varying the amount of leucylglycylglycine are given in Fig. 1; these show that the concentration used by Fruton (1946) is approximately maximal.

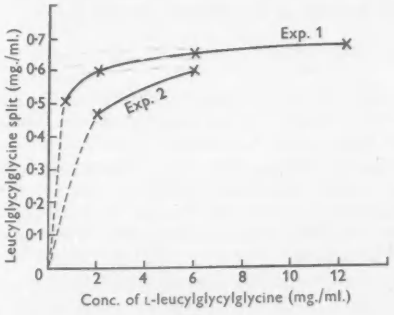

Fig. 1. Effect of substrate concentration. pH 7·9, 38°. Acetone-dried skin extracted with 2% NaCl. Extract mixed with equal volume of DL-leucylglycylglycine solution and incubated for 2 hr. Estimations by titration.

Metals. The results of similar experiments on the variation of activity with varying concentration of manganese are shown in Table 9, and indicate an optimal concentration of 0·002 M-Mn; it is to be noted that Fruton used 0·001 M and Berger & Johnson (1939) found that the leucylpeptidase activity of their extracts of hog intestinal mucosa increased steadily as the concentration of Mn increased from 10^{-6} to 10^{-2} M.

Table 9. *Effect of manganese concentration on leucylglycine activity*

(9·4 mg. DL-leucylglycine/ml.; 2 hr. incubation; pH 7·9.)

Exp. no.	$MnSO_4$ (M)	Leucylglycine split (mg./ml.)
1	Nil	0·37
	0·0002	0·80
	0·0006	1·19
	0·001	1·33
	0·002	1·45
	0·005	1·35
2	Nil	1·18
	0·001	4·02
	0·002	4·17

(Experiments 1 and 2 were made with different skin extracts.)

DISCUSSION

Comparison of these results upon rat skin with those of Fruton (1946) upon rabbit skin show that the skins differ in the proportions present of the two peptidase activities investigated.

Since the Fruton-type extracts contain the skin proteinase, though in weak concentration, it can be concluded from Fruton's negative results with carbobenzyloxy-L-glutamyl-L-tyrosine and carbobenzyloxyglycyl-L-phenylalanine, that the skin proteinase differs from pepsin and carboxypeptidase, and therefore from any of the hitherto recognized groups of proteolytic enzymes. Since, also, it has

recently been shown (Peters & Wakelin, 1948; Grob, 1946) that trypsin and chymotrypsin are inactivated by thiol compounds, whereas the skin proteinase is not, the conclusion stands firm at present that it is a proteinase of different type.

SUMMARY

1. The proteinase present in acetone-dried extracts and in Fruton-type extracts of fresh skin from the rat have been compared and further characterized in the light of Fruton's results.

2. The acetone-dried extracts have high proteinase activity and low dermo- and amino-peptidase activity with only slight activation effects of manganese, whereas the fresh skin extracts have a high peptidase and low proteinase activity.

3. The skin proteinase differs from previously recognized enzymes.

4. Manganese activates the leucine aminopeptidase optimally at 0·002 M; cobalt and magnesium also activate.

We are indebted to Prof. K. Linderstrøm-Lang for his hospitality and kindness in showing his technique; also to the Nuffield Committee and to the Osler Trustees (Oxford) for grants in aid of this work.

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Separation and Estimation of Saturated C_2–C_8 Fatty Acids by Buffered Partition Columns*

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The lack of adequate small-scale methods for the analysis of mixtures of fatty acids has long been felt. Following up an observation of Lester Smith (1942), Elsden (1946*a*) has recently introduced an important new technique, based on partition chromatography, for the separation and estimation of the lower members of the saturated series of fatty acids. A wet silica gel was impregnated with bromocresol green, a small sample of the mixture of acids, dissolved in chloroform, was introduced at the top of the column and, after its penetration into the gel had taken place, a suitable mixture of butanol and chloroform was allowed to percolate through the column. By using the colour change of the indicator as a pointer to the positions occupied by the acids, Elsden was able to estimate propionic and butyric acids. Under certain conditions valeric acid also could be estimated, but the precise behaviour of the gel is conditional upon its properties. Acetic acid could not be estimated except by difference. The method has been used by Elsden (1946*b*) and by Elsden, Hitchcock, Marshall & Phillipson (1946) with excellent results in the analysis of rumen contents, but it has certain disadvantages. Different batches of silica prepared in the same manner often differ considerably in their properties, and of several batches only one may be suitable for use. The range of the method is very limited: acetic acid can only be estimated by difference and the method cannot ordinarily be applied to acids higher than butyric; such higher acids fail to undergo separation and can only be determined collectively. Finally, no separation is possible between the isomers of any given acid.

We have now devised a procedure which obviates most of the disadvantages of Elsden's method and are able to identify, separate and estimate most of the naturally occurring, steam-volatile members of the saturated fatty acid series with a high order of accuracy and recovery. The use of indicators on the column is dispensed with, acetic acid can be determined directly, small differences in the properties of the silica are of no consequence, and almost complete resolution of certain isomeric valeric acids has been achieved.

* A preliminary account of this work was communicated by one of us (R. S.) to the Biochemical Society on 31 October 1947 (Scarisbrick, Baldwin & Moyle, 1948).

In order to extend the range of the method it was necessary to find conditions which would increase the relative solubility of the higher acids in the stationary, aqueous phase. We tried the use of alkaline columns with the idea of altering the effective partition coefficient in favour of the aqueous phase, and even in our earliest experiments we were able to achieve virtually complete separations of caproic and valeric acids (Fig. 1). It was

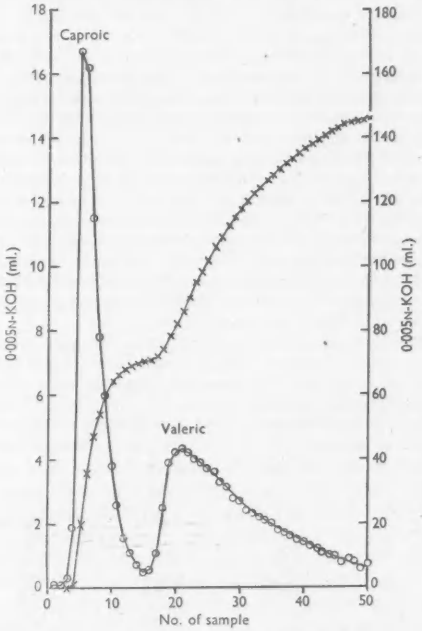

Fig. 1. Separation of caproic and valeric acids. Column: 5 g. silica, 0·5 ml. litmus, 2·5 ml. 2·5M-K_2HPO_4, 0·34 ml. 10N-KOH. Solvent: chloroform. Ordinates: titres of successive 5 ml. samples of chloroform elutriates, o individual (scale on left), × aggregate (scale on right).

hoped that by means of even more alkaline columns still higher acids would prove to be separable but, unfortunately, as the length of the fatty chain is increased, the solubility of the soaps decreases rapidly and sets an eventual upper limit to the range of the method; lauric (C_{12}) and higher acids cause blockage of the columns. It is necessary, therefore, to effect a preliminary separation of the lower acids from lauric acid and higher members of the series; this can be done satisfactorily by steam distillation by the method of Friedemann (1938) whereby formic acid, if present, is destroyed. The free, steam-volatile acids are then recovered in solution in a suitable mixture of chloroform and butanol by a procedure similar to that of Elsden (1946*a*, *b*).

Throughout our experiments we have used columns heavily buffered with phosphate. Neither this nor the employment of alkaline gels is entirely novel (see Synge, 1946, for further references) but, so far as we are aware, columns of the type used here have not been previously used for the same purpose, although Sato, Bary & Craig (1947) have used buffers in a method based on 'counter-current distribution' which allows the separation of fatty acids as far as valeric. Goodall & Levi (1946) have also used buffered chromatograms in a micromethod for the separation of the various types of penicillins on filter paper.

EXPERIMENTAL

Reagents

Solvents. Chloroform, previously washed with water and dried over $CaCl_2$, and *n*-butanol are redistilled in an all-glass apparatus. Normally we use mixtures containing 1, 10 and 30% (v/v) of *n*-butanol in chloroform (referred to in the text as '1% butanol', etc.), all of which are equilibrated by shaking with 2M-K_2HPO_4. It is advisable to filter the equilibrated solvents through dry paper before use.

Silica. Silica may be prepared according to the directions given by Gordon, Martin & Synge (1943) or Isherwood (1946). Several different preparations were used in the course of these experiments but, as we shall show, there is little if anything to choose between different samples.

Buffers. Stock 2M solutions of KH_2PO_4, K_2HPO_4 and K_3PO_4 are required and should be made up from the purest specimens available. We found that a commercial sample of K_3PO_4 gave very poor recoveries compared with a specimen made by adding the theoretical amount of A.R. KOH to A.R. KH_2PO_4. From these stocks the following standard buffers are prepared: I, 2 vol. K_2HPO_4+1 vol. KH_2PO_4; II, 2·5 vol. K_2HPO_4+3·5 vol. K_3PO_4; III, K_3PO_4 alone; IV, KH_2PO_4 alone. These are referred to as 'buffers I, II', etc.

General procedure

Columns. Five g. silica are intimately mixed with 3 ml. of the appropriate buffer by grinding in a small mortar. The mass is suspended in about 40 ml. 1% butanol and poured through a funnel into a tube of the usual design (a drawing of the apparatus actually employed is reproduced in Fig. 2), and the tube refilled with 1% butanol by means of a separating funnel with a bent outlet (Fig. 2*A*). The solvent is then allowed to percolate through the column for 20–30 min. to permit proper packing of the gel and the formation of a firm surface.

Introduction of acids. The acids to be analyzed are introduced in the form of a solution, usually in 1% butanol. Samples as large as 50 ml. may be taken if necessary, but for most purposes 1–5 ml. is convenient. When small samples are to be introduced the device illustrated in Fig. 2*B* is useful: the delivery tube is placed in position with its tip a few millimetres above the surface of the gel. As soon as the last of the solvent has drained into the gel the sample is cautiously run in through the delivery tube. At the same moment the tap (*T*) is closed and the automatic siphon (*S*) placed in position with a sampling tube

below. The tap (*T*) is then reopened. As soon as the sample has drained into the gel the delivery tube is washed down twice with about 1 ml. 1% butanol and then withdrawn. The separating funnel, already charged with 1% butanol, is placed in position. As soon as the washings have entered the gel the tap of the funnel is carefully opened and the solvent admitted to the column.

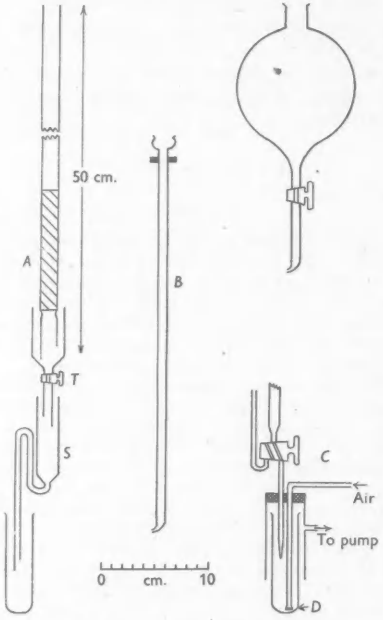

Fig. 2. Apparatus (see text).

Sampling. Successive, approximately equal samples of about 5 ml. (the exact volume is immaterial) are collected in dry tubes (4 in. × 1 in.) by means of the automatic siphon (*S*). Each sample is titrated in turn and the titres, after correction for blanks, are plotted against the number of samples. It is usually convenient to plot the titres both individually and cumulatively.

Titrations. To avoid the inconvenience of titrating in a heterogeneous medium 0·005 N-KOH is used in CO_2-free methanolic solution. The solution is stored in a bottle guarded against CO_2 and delivered by gravity to a guarded, self-filling 10 ml. micro-burette, the stopcock of which is lubricated with a silicone preparation.

A stream of dry, CO_2-free air is passed through the sample throughout the titration, using a small sintered glass distributor (*D*) to obtain a stream of fine bubbles. The air should be passed for at least 30 sec. before beginning to titrate; shorter periods of preliminary gassing fail to remove CO_2 while long periods may lead to loss of acids by volatilization. The butanol, chloroform and methanol vapours are sucked away by the pump (see Fig. 2*C*).

Indicator and end point. In our early experiments we used phenolphthalein as indicator, as is usually advised, but we later abandoned this in favour of cresol red. A 0·04% solution in methanol is used, two drops being taken for each 5 ml. solution undergoing titration. The colour changes from yellow through a brownish red and finally, very sharply indeed, to a bluish red: even when 0·005 N-alkali is being used, 0·01 ml. suffices to produce the final change of colour, which is more easily seen by daylight than by artificial illumination.

Cresol red has certain disadvantages which, however, can easily be overcome, and are compensated for by the sharpness of the end point. It is preferentially soluble in water so that the samples for titration must be collected in dry tubes, and the air stream must be dried. There is a slight lag in the establishment of the final end point, especially in 30% butanol, but this can be overcome by waiting for 30 sec. after the end point has apparently been reached in order to see whether the brownish red colour returns. There is a further and more subtle difficulty. If, when the total titre in 1% butanol is less than about 0·5 ml., a dropwise titration is carried out, a false end point may be reached with the addition of a few drops of the alkali. On the addition of a further 1–2 drops the indicator again turns yellow, and only then begins to approach the true end point. This curious phenomenon, which is not observable in 10 or 30% butanol, can be avoided by the addition of a few drops of methanol before starting to titrate samples in which a small titre is expected.

Blanks. Blank values are determined by running samples of the solvents through virgin columns. They vary slightly from one sample of silica to another, but the errors introduced by these variations into the final estimates are of the order of not more than 1% when total titres of 10 ml. or more are being handled. In our own experiments the following average blanks were found:

	Blank (ml. 0·005 N-KOH/5 ml. sample)		
Buffer	1%	10%	30%
I	0·023	0·034	0·036
II	0·024	0·032	0·038
III	0·022	0·034	0·036

Change of solvent. When one acid has been eluted from the column the elution of the next can be hastened by replacing the solvent by another containing more butanol. Generally speaking, the exchange is made when the titre has dropped to the blank level or as soon as an increase of at least 0·1 ml. in titre is observed.

General operation. It is usually convenient to collect samples every 4–5 min. The rate of flow diminishes with increasing concentrations of butanol and with increasing pH, but depends mainly upon the porosity of the particular sample of silica in use. It can be controlled by modifying the proportions of silica and buffer in the mixture, but it is better to rely upon alterations in the hydrostatic pressure of solvent above the column. It is also possible to mix samples of different porosities together in order to obtain suitable rates of flow.

An experimental run can be interrupted, if necessary, by fitting to the top of the tube a rubber bung pierced by a small stopcock. The tube is filled very nearly to the top with solvent, the bung placed securely in position and the tap closed; in this way it is possible safely to discontinue operations for several hours.

Table 1. *Behaviour of aliphatic acids* (C_2 *and upwards*) *on columns I, II and III*

Column	pH*	% butanol	Acids: Eluted	By-passed†	Retained
I	6·6	1	C_4	C_5 and higher	—
		10	C_3		
		30	C_2		
II	8·4	1	C_6	C_7 and higher	C_3, C_2
		10	C_5		
		30	C_4		
III	9·5	1	C_7 and higher	—	C_4 and lower
		10	C_6		
		30	C_5		

* Determined by glass electrode (16°) on mixtures of 5 g. silica with 3 ml. buffer suspended in 10 ml. water: these figures are more useful as a check on the properties of the silica than as indications of the operative pH of the columns.
† In the first five to six samples.

Operational columns. It is not possible to carry out complete separations of more than three acids upon any one column under the conditions we employ, and we have therefore developed three types of columns buffered, respectively, with buffers I, II and III. These columns, referred to as 'columns I, II and III', cover the whole useful range of the method: their performances are summarized in Table 1 (see also Figs. 3–5).

RESULTS

Separation and recovery of acids

Recovery experiments were first carried out on carefully redistilled specimens of single acids, the total load taken being of the order of 20 ml. 0·005N in each case. The recoveries are listed in Table 2. Feeling satisfied that virtually quantitative recoveries of single acids can be achieved, we prepared a series of mixtures of known acids and put them through the usual procedure. The results (Table 3, Figs. 3–5) showed that virtually complete separations of naturally occurring acids are possible up to C_8, and that quantitative recoveries of the separated components can be achieved. Oenanthic acid (C_7) is separable from caproic (C_6) but not from caprylic acid (C_8); pelargonic acid (C_9) is inseparable from caprylic (C_8) and higher acids.

Behaviour of isomers. *n*- and *iso*Butyric acids behave so similarly on the columns that they are indistinguishable and the same is true of *n*- and *iso*caproic acids. We made a special study of the valeric acids, viz. *n*- and *iso*valeric, trimethylacetic and DL-methylethylacetic acids.

Since sharper separations are normally found between acids that come late through the columns (see Figs. 3–5) we studied the behaviour of the isomers on columns of type III, from which they are eluted only by 30% butanol. Each experiment was performed on a separate column and all the columns were prepared in the same way and under identical conditions. It was found that by increasing the relative proportion of buffer to silica better separations were obtained: these columns were therefore made from 4 g. of silica and 3 ml. of buffer III. The results showed that *n*-valeric and trimethylacetic acids behave almost identically, while *iso*valeric and DL-methylethylacetic acids come through together but considerably later than the other two. We prepared an equimolecular mixture of the *n*- and *iso*-acids and examined this on one of these columns. The resulting curve (Fig. 6A) showed two well-separated peaks, indicating that a partial separation of the two isomers had been accomplished. Similar mixtures of trimethylacetic and DL-methylethylacetic (Fig. 6B), *n*-valeric and trimethylacetic (Fig. 6C) and *iso*valeric and trimethylacetic acids (Fig. 6D) were similarly treated and again gave partial separations.

Although we have not succeeded in accomplishing total separations even in these favourable cases, it is, nevertheless, established that *n*-valeric and trimethylacetic acids can easily be distinguished from *iso*valeric and DL-methylethylacetic acids.

Influence of some experimental conditions

Properties of the silica. Several different samples of silica were used in the course of our work. These included one too acid and another too alkaline for use in Elsden's (1946*a*) procedure, two more that gave good results by his method, and a sample of Isherwood's (1946) non-adsorbent silica. Virtually identical results were obtained with all samples.

Fig. 7 presents the results obtained by analyzing samples of the same mixture of acids (*n*-caproic, *iso*valeric and *n*-butyric acids) on four different silicas. In examining these curves the following facts must be borne in mind. The precise position taken up by a given acid is influenced to some extent by the rate of flow of the column and therefore by the porosity of the gel. This is shown by slight differences in the positions of the first bands in Fig. 7. More important, however, are the differences due to the fact that the butanol content of the

Table 2. *Recovery of single acids*

No. of C atoms	Acid	Column	Amount taken (ml. 0·005 N)	Amount recovered (ml. 0·005 N)	Recovered (%)
2	Acetic	I	20·6	20·6	100
3	Propionic	I	18·8	18·2	97
	Propionic	I	18·8	18·4	98
4	*n*-Butyric	I	19·6	19·4	99
	n-Butyric	II	19·3	18·8	98
	*iso*Butyric	I	21·5	21·4	99
	*iso*Butyric	II	21·5	20·7	96
5	*n*-Valeric	II	18·5	18·3	99
	n-Valeric	II	18·5	17·7	96
	*iso*Valeric	II	19·6	20·0	102
	*iso*Valeric	III	19·6	19·2	98
	Trimethylacetic	II	19·9	20·6	103
	Trimethylacetic	III	19·9	19·7	99
6	*n*-Caproic	II	17·3	17·3	100
	n-Caproic	III	19·3	19·1	99
	*iso*Caproic	III	19·2	19·3	101
7	*n*-Oenanthic	III	18·8	18·3	98
8	*n*-Caprylic	III	19·3	19·2	99
9	*n*-Pelargonic	III	19·2	19·2	100
10	*n*-Capric	III	18·6	18·5	99

Table 3. *Recovery of acids from mixtures*

Column	Acids taken	Amount taken (ml. 0·005 N)	Amount found (ml. 0·005 N)	Recovered (%)	Total recovered (%)
I	*n*-Butyric	19·6	20·0	102	101
	Propionic	18·8	17·9	96	
	Acetic	20·6	21·4	104	
II	*n*-Caproic	17·8	17·8	100	99
	n-Valeric	18·5	18·6	100	
	n-Butyric	19·3	18·6	97	
II	*n*-Caproic	17·8	18·0	101	98
	n-Valeric	18·5	18·6	100	
	n-Butyric	19·3	18·9	98	
II	*n*-Caproic	17·8	18·6	104	101
	n-Valeric	18·5	18·9	102	
	n-Butyric	19·3	18·8	98	
III	*n*-Capric	18·6	37·7	100	99
	n-Caprylic	19·3			
	n-Caproic	19·3	19·0	99	
III	*n*-Capric	18·6	37·8	100	99
	n-Caprylic	19·3			
	n-Caproic	19·3	18·8	98	
III	*n*-Capric	18·6	37·5	99	100
	n-Caprylic	19·3			
	n-Caproic	19·3	19·4	100	

developing solvent was not increased at the same time in all cases. Such an increase results in a sharp rise in titre as the next acid comes through, and any delay in changing the solvent is naturally followed by a later rise in the curve. When allowances are made for these factors, the differences between the four curves become insignificant, indicating that it is unnecessary to take elaborate precautions in preparing the silica and, incidentally, that different samples of silica can safely be mixed together in order to obtain columns running at convenient speeds.

Effect of concentration of buffer. We adopted the use of 2M buffers as standard procedure. Fig. 8 shows the effects of using more dilute buffer on columns of type II. Using M instead of the usual 2M phosphates the separation between the first two acids (*n*-caproic and *iso*valeric) is no longer com-

plete, though the separation between the second pair (*iso*valeric and *n*-butyric) is still very good. In 0·5M buffer the second separation is also impaired. Essentially similar results were obtained with the other columns.

sensitive to loads of 0·01 mmol. or less, so that small amounts of one acid can be detected and estimated in the presence of larger amounts of others. Excellent separations can be obtained with loads up to about 1 mmol. of each acid by the use of columns

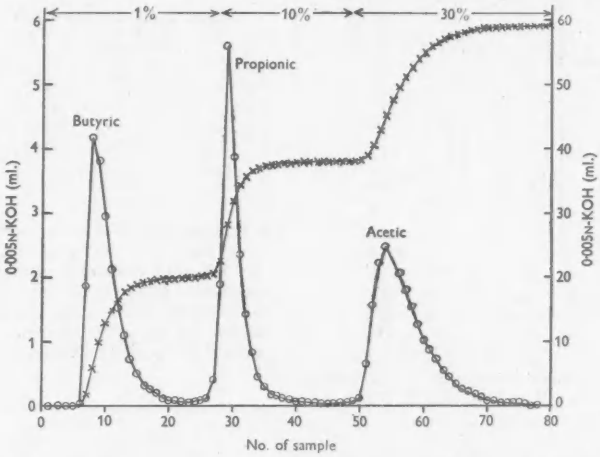

Fig. 3. Separation of *n*-butyric, propionic and acetic acids (column I). The butanol content of the developing solvent was changed at the points indicated. Acids: approx. 1 ml. 0·1N of each acid. Ordinates: titres of successive samples, o individual, × aggregate.

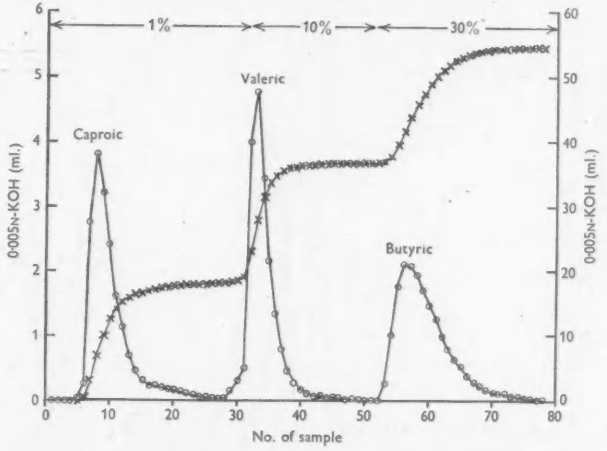

Fig. 4. Separation of *n*-caproic, *n*-valeric and *n*-butyric acids (column II). Details as for Fig. 3.

Effect of load. The optimal load for the columns is about 0·1 mmol. (≡20 ml. 0·005N) of each acid in the mixture, corresponding to about 6 mg. acetic or 10 mg. valeric acid. The method is, however, still

of similar length, but about five times as large a cross-sectional area.

Volume of sample. In the method of Elsden (1946*a*) it is necessary to introduce the acids to be analyzed

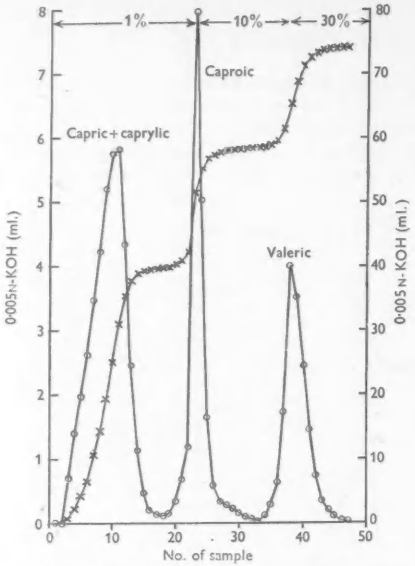

Fig. 5. Separation of *n*-capric+*n*-caprylic, *n*-caproic and *n*-valeric acids (column III). Details as for Fig. 3.

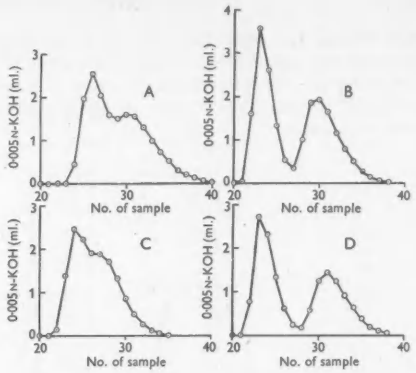

Fig. 6. Behaviour of isomeric valeric acids (column III). A, 0·5 ml. 0·1 N-*n*-valeric + 0·5 ml. 0·1 N-*iso*valeric acids. B, 0·5 ml. 0·1 N-trimethylacetic + 0·5 ml. 0·1 N-DL-methylethylacetic acids. C, 0·5 ml. 0·1 N-*n*-valeric + 0·5 ml. 0·1 N-trimethylacetic acids. D, 0·5 ml. 0·1 N-trimethylacetic + 0·5 ml. 0·1 N-*iso*valeric acids. Solvent: 30% butanol in chloroform throughout.

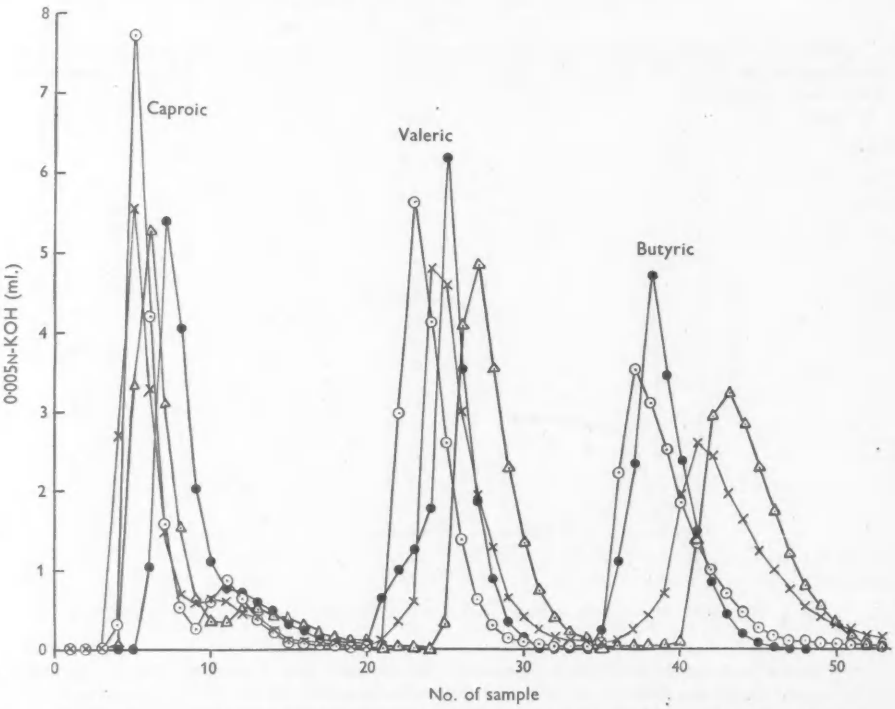

Fig. 7. Analysis of a mixture of *n*-caproic, *iso*valeric and *n*-butyric acids (column II). Four equal samples of the same mixture were separately analyzed on four different samples of silica. Note: the solvents were not changed at the same point in each case. Acids: approx. 1 ml. 0·1 N of each acid.

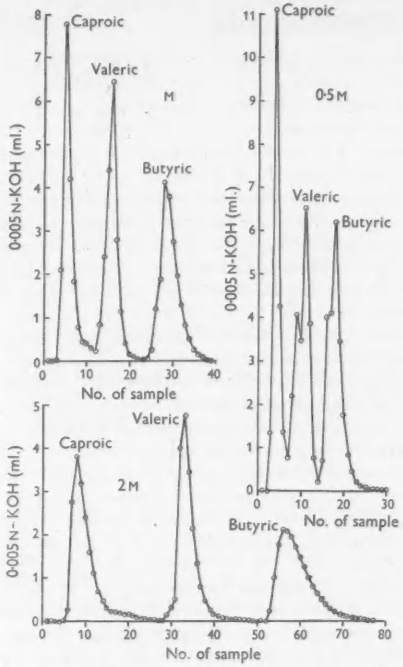

Fig. 8. Influence of concentration of buffer on separation of *n*-caproic, *iso*valeric and *n*-butyric acids on column II. Acids: 1 ml. 0·1 N approx. of each acid.

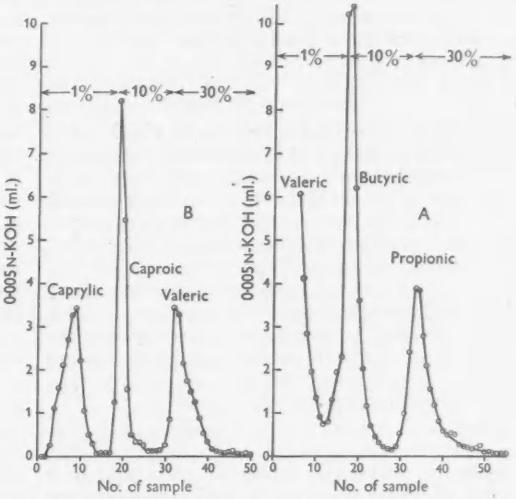

Fig. 10. Approximate analysis of a mixture on two columns (see text). Sample, 1 ml. A, Column IV; the first five samples were massed and placed on B (column III).

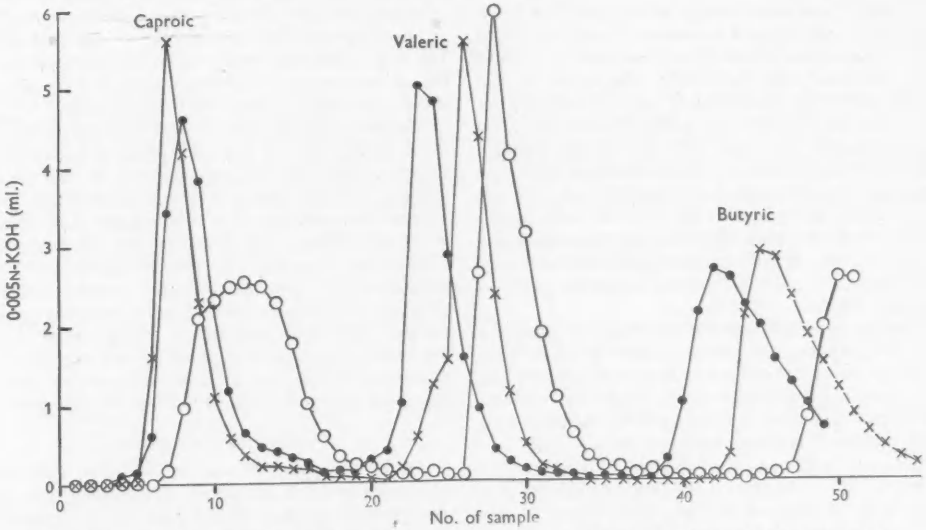

Fig. 9. Effect of volume of sample (column II). Separation of *n*-caproic, *n*-valeric and *n*-butyric acids. Acids: equivalent of 1 ml. 0·1 N approx. of each acid. Volume of sample: o, 50 ml.; •, 20 ml.; ×, 10 ml.

in a total volume of not more than 3 ml. With the 5 g. buffered columns, even when samples of 50 ml. are taken, there is still excellent resolution, though the first band is considerably broadened (Fig. 9).

Applications of the method

Identification and approximate assay of components of a mixture. Each acid shows strongly characteristic behaviour on the columns. Thus the valeric acids are by-passed in the first five to six samples of 1 % butanol by column I, eluted rapidly by 10 % and only very slowly by 1 % butanol from column II, and rapidly eluted only by 30 % butanol from column III. So characteristic is the behaviour of each member of the series of acids studied that it may be taken as evidence of identity, with the reservation that only certain pairs of the isomers we have tested proved to be distinguishable (p. 311), even under carefully controlled conditions.

Before proceeding to exact analysis it is often desirable to carry out an approximately quantitative analysis of an unknown mixture in order to discover what acids are present and in what concentrations. This can be done by taking advantage of the large sample volumes that the columns can handle. For this purpose an additional column (IV) is of great service: this is buffered with 2 M-KH_2PO_4 alone, and by-passes caproic and higher acids, yields up valeric with 1 %, butyric with 10 % and propionic with 30 % butanol. Acetic acid is retained but can be approximately assayed by difference.

A small, measured sample of the mixture to be analyzed is placed on a column of type IV and the first five samples, which contain some of the valeric acid together with practically the whole of the higher acids, are not titrated but massed together and reserved. The column is then developed in the usual manner. A column of type III is then set up, the five reserved samples are placed upon it and the column then developed. The results of such an experiment are shown in Fig. 10: the acids taken in this case consisted of a 1 ml. sample containing about 0·1 mmol. each of acetic, propionic, *n*- and *iso*butyric, *n*- and *iso*valeric, *n*-caproic and *n*-caprylic acids.

Only approximate results can usually be obtained in this manner, but the procedure gives reliable indications of the nature of the acids present and approximate values for their respective concentrations. It is then possible to select columns suitable for exact analysis under optimal conditions.

Routine analyses of mixtures. Where routine analyses are to be carried out, and the identities of the acids present have been established, it may not be necessary to employ all three of the standard columns. In the analysis of rumen contents, for example, in which only acetic, propionic and butyric acids are usually assayed (Elsden, 1946*b*; Elsden *et al.* 1946, for example), only column I will be required. In a hypothetical case where, say, only valeric, butyric and propionic acids were to be determined, a column intermediate between types I and II could be specially designed for the purpose. It is reasonable to assume that intermediate columns would yield results as quantitative as those obtained on the standard columns used in the present experiments. In addition, higher acids by-passed by one column might be put directly on another that is more alkaline.

Detection and estimation of impurities: purification of acids. As the method is sensitive even to very small loads, it is not difficult to detect the presence of traces of contaminants in supposedly pure specimens of acids. We were able, for example, to demonstrate the presence of approximately 4 % of a valeric and 6 % of propionic acid in a redistilled sample of 'pure' *iso*butyric acid. In another experiment an allegedly pure specimen of *n*-caproic acid was found to contain no less than 17 % of an unknown impurity.

Since the columns can handle loads up to about 0·2 mmol. of acid, specimens of this order can be freed from traces of impurities by passage through an appropriate column and subsequent recovery by neutralization and evaporation to dryness.

Preparation of characteristic derivatives. Considerable amounts of purified acids can be obtained by the procedure outlined in the preceding paragraph and are of great value for the preparation of characteristic derivatives, such as the piperazonium salts or phenylhydrazides, for purposes of identification. The main difficulty likely to be encountered here lies in the removal of the indicator, but we avoid this by proceeding in the following manner.

A suitable column is set up and loaded with about 0·2 mmol. of each of the acids to be characterized. Samples are collected and titrated in the usual manner until the first acid begins to come through. The next six samples, which contain the bulk of this acid, are massed together, without titration, for subsequent recovery. Further samples are then titrated until the second acid begins to come through and this is likewise collected in six samples, again without titration, and so on. When more than 0·2 mmol. of an acid is required it is an easy matter to run more than one column and mass the corresponding eluates or to work with wider columns.

Limitations of the method

As the foregoing discussion indicates, this new method possesses numerous advantages over its predecessors. Certain drawbacks remain, however, in particular the inability of the columns completely to resolve mixtures of isomeric acids and of acids higher than caprylic.

It seems to be well established (Hilditch, 1947) that, with the exceptions of propionic and *iso*valeric acids, fatty acids containing either a branched chain or an odd number of carbon atoms occur but rarely in nature. Our procedure is therefore capable of separating and analyzing all the naturally occurring steam-volatile saturated fatty acids as far as C_8. We have shown that oenanthic (C_7) acid can be completely separated from caproic acid (C_6) but not from caprylic (C_8), while pelargonic (C_9) is inseparable from caprylic (C_8) and capric (C_{10}). While for biochemical purposes this limitation is perhaps not a serious one, for purely chemical work, in which odd-numbered acids are more likely to be encountered, the present method will break down in cases where acids containing more than seven carbon atoms have to be dealt with.

SUMMARY

1. A new method is described for the identification, separation and estimation of most of the steam-volatile members of the saturated series of fatty acids. The procedure is based on the principle of partition chromatography: heavily buffered silica gels, without indicator, form the stationary phase of the partition columns, the moving phase consisting of mixtures of chloroform and butanol.

2. Naturally occurring fatty acids from acetic to caprylic can be identified, quantitatively separated and estimated. Caprylic cannot, however, be separated from higher acids.

3. Of the non-natural acids, oenanthic can be separated from lower but not from higher acids.

4. Partial separation has been achieved between isomeric forms of valeric acid but isomeric acids cannot in general be distinguished or separated.

5. Acids can be recovered from the columns in quantities large enough to permit the preparation of characteristic derivatives.

6. Optimal results are obtained with quantities of the order of 0·1 mmol. of each acid, whether taken separately or in mixtures.

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Note. The range of the method has been improved by the use of a column buffered with 9 parts of 2 N-KOH to 1 part of a 2 M-solution of glycine and KCl. Caprylic (C_8) and higher acids are eluted with 1 % butanol, oenanthic (C_7) with 10 % butanol and caproic (C_6) with 30 % butanol.

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Experimental Evidence that the Stem-end Blackening Pigment of Potatoes is a Compound of Iron

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In studies of the environmental conditions of growth which lead to the production of potatoes liable to show stem-end blackening, the quantity of pigment present was estimated by visual judgement (Wager, 1946, 1947). In the course of experiments to develop an alternative, physical method for the estimation of the stem-end blackening pigment, it was found that the intensity of the colour of the extracts was increased by the addition of small amounts of iron salts, and further work was directed to this point.

Robison (1941) suggested that the pigment might be a compound of iron. The evidence given was concerned with the relationship between the iron content of the tubers and the amount of stem-end blackening shown by them, and was conflicting. She also suggested that the blue-black compound was an oxide of iron. These views were criticized by Cowie (1941). Nutting (1942), on the basis of analogy with other pigments, suggested the possibility that the blue-black pigment was a metallic complex, but offered no supporting evidence.

METHODS

The potato extracts were prepared from median longitudinal slices, 3 mm. thick, of peeled tubers: 40 or 50 potatoes were sliced to obtain a composite sample. The slices were washed free of surface starch, cooked in glass-distilled water, reduced to a sludge and left to stand for about 1 hr. to allow time for equilibration of soluble material between the potato cells and the liquid. The potato cells were then filtered off and the cloudy filtrate left to stand with chloroform for several days, during which time precipitation occurred. One or two successive filtrations gave an extract with only a trace of opalescence.

The stem-end blackening pigment is sensitive to change in acidity, being colourless or nearly so at an acidity of low pH (3 or less), and attaining a maximal intensity of colour at one of pH 8–9 (Wager, 1945). This change in colour was the basis of the method of estimating the pigment. The absorption of light by the solution was measured at pH 7·40 and also at about pH 3, and the difference between these two values was used as an estimate of the content of stem-end blackening pigment. This estimation was fairly specific, but in some cases there may have been a slight change in the degree of opalescence of the solution on acidification, and it seems probable that in all extracts there were small amounts of compounds which were pale yellow in alkaline solution and colourless in acid solution; such compounds could be seen in some of the extracts containing little stem-end blackening pigment.

Iron was estimated by $\alpha\alpha'$-dipyridyl using the potato extract prepared as above (i.e. without ashing). The development of colour was slow; 3–4 hr. were required for maximal intensity, whereas in pure solutions of iron 15 min. only. The average recovery of iron added to the solution was within about 2% of the value shown by the calibration curve, but individual values showed some experimental error. The complete recovery of amounts of iron as low as 1 μg./ml. from solution with 3–4 times this amount of iron, as determined by the $\alpha\alpha'$-dipyridyl reagent, suggests that all the ionizable iron present was estimated, and this has been assumed.

pH was determined with a glass electrode, and light absorption by a photometer (Hilger Spekker) using a violet filter, Ilford no. 601.

The samples of potatoes used in this work were all commercially grown in East Anglia. They came from a variety of soil types and consisted of five samples of King Edward, five of Doon Star and eleven of Majestic potatoes. (The author's thanks are due to Mr B. S. Smith of the Potato and Carrot Division of the Ministry of Food, Peterborough, who arranged for the collection of these samples.)

RESULTS

Extracts prepared from tubers liable to stem-end blackening are grey in colour at pH 6 and become somewhat browner as the pH is adjusted to 7·5. The addition of a solution of an iron salt, which increases the iron concentration by a few μg./ml., leads to a marked increase in intensity of colour. This increase in intensity of colour may be explained by the assumption that the stem-end blackening pigment is a compound of a colourless precursor and iron, and that there is normally an excess of the colourless precursor. On the other hand, there might be present two pigments, the stem-end blackening pigment and another which was affected by iron, e.g. tannin. To test whether tannin was present an extract with a high intensity of colour was shaken at intervals with droplets of gelatin for 2 hr. The gelatin was filtered off and melted, and then to the diluted and melted gelatin and to the filtrate was added an excess of iron (10 μg./ml.). The change in intensity of colour with pH was determined for both solutions. The gelatin contained slightly less material that increased in colour with added iron than did the

filtrate, i.e. there was no evidence of concentration in the gelatin. The same technique when applied to a solution of tannic acid of similar concentration resulted in a marked concentration of tannic acid in the gelatin. As a further test for the presence of tannin an attempt was made to absorb the pigment on goldbeater's skin. This was also negative. There is, therefore, a prima facie case for assuming that the increase in colour with iron is not due to the presence of tannins.

Extracts were prepared from different samples of potatoes and their pH was adjusted to 7·40. To 10 ml. portions of these extracts 1–15 μg./ml. of iron was added as ferrous sulphate. The concentration of iron and also the amount of stem-end blackening pigment were then determined, with the results shown in Fig. 1. The lowest points on the curves are for the extracts with no added iron.

The colour of any given extract is clearly dependent on the concentration both of iron and of the colourless precursor of the stem-end blackening pigment. The maximum intensity of pigment obtained with a high concentration of iron may be taken as a measure of the total amount of stem-end blackening pigment present. This has varied in different extracts from 6 to 31 units, whilst the concentration of iron in the extract merely varied from 1·4 to 3·5 μg./ml. and could not be correlated with the variation in stem-end blackening pigment. It follows, therefore, that the major factor in determining the amount of blackening that develops in potatoes after cooking is the content of the stem-end blackening pigment precursor and not the content of iron (cf. Robison, 1941). The relationships between the content of iron, the content of stem-end blackening pigment, and the variety and conditions of growth of the tuber will be discussed in a subsequent paper.

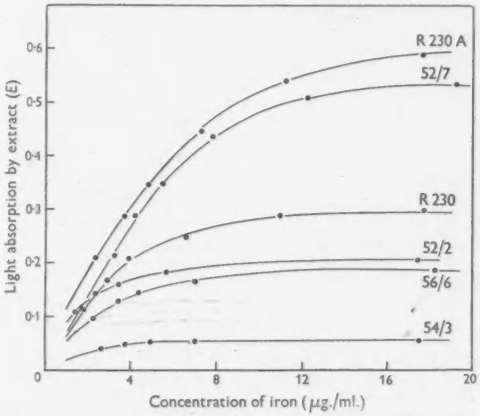

Fig. 1. The relationship between the estimated content of iron in potato extracts and the concentration of stem-end blackening pigment, expressed as the optical density of the extract.

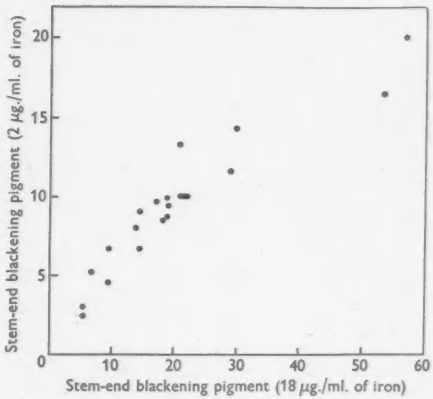

Fig. 2. The concentration of stem-end blackening pigment, expressed as optical density, in potato extracts at a low concentration of iron, 2 μg./ml., plotted against the concentration of pigment in the same extract at a high concentration of iron, 18 μg./ml.

If all these curves, when extrapolated backwards, had passed through the origin it would have been clear evidence that the stem-end blackening pigment was a compound of iron. Some of the curves, however, when extrapolated, do not appear to pass through the origin, and it seems reasonable to assume that in these cases there were present compounds, sensitive to the change in pH, other than the stem-end blackening pigment: a faint yellow colour at pH 7·4, which was lost in acid solution, was noticed in some extracts which contained very little stem-end blackening pigment. The curves in Fig. 1, therefore, offer fair evidence for the combination of a colourless precursor with iron to give the stem-end blackening pigment.

If these extracts contained coloured stem-end blackening pigment and a different colourless compound, liable to darken with iron, it would be very unlikely that the concentration of both compounds would always be in the same ratio. Unless this were so the intensity of colour developed at a high concentration of iron would bear no constant ratio to that at a low concentration. These two values have been plotted against each other in Fig. 2 for all extracts prepared, and a sensibly constant ratio between them is clearly shown. Some small divergencies from a constant ratio must be expected in view of the experimental errors and of the presence of other light-absorbing compounds in some of the

extracts. The view that the extracts contained only one main compound that combines with iron, and that this combination gives rise to the stem-end blackening pigment receives support, therefore, from Fig. 2.

A simple but very conclusive visual demonstration of the combination of the stem-end blackening pigment with iron may be made by immersing one half of cooked tubers, showing stem-end blackening, in a solution of 10 μg./ml. of iron for about 1 hr. and immersing the opposite halves in distilled water as a control. The iron causes a great intensification of colour in regions previously blackened, but a negligible discoloration occurs in the rest of the tuber.

SUMMARY

Evidence is offered to support the view that the stem-end blackening pigment of potato is a compound of iron with a colourless precursor. The concentrations of iron and of the precursor vary in different samples of potatoes. The range of concentration of the precursor is greater than that of iron.

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